

GRAS Notice (GRN) No. 510

<http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm>

ORIGINAL SUBMISSION

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April 3, 2014

VIA FEDERAL EXPRESS

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notification for Acid Lactase from *Aspergillus oryzae* Expressed in *Aspergillus niger*

Dear Sir or Madam:

On behalf of DSM Food Specialties ("DSM"), we are submitting under cover of this letter three paper copies and one eCopy of DSM's generally recognized as safe ("GRAS") notification for its acid lactase from *Aspergillus oryzae* expressed in *Aspergillus niger*. The electronic copy is provided on a virus-free CD, and is an exact copy of the paper submission. DSM has determined through scientific procedures that its acid lactase enzyme preparation from *Aspergillus oryzae* expressed in *Aspergillus niger* is GRAS for use in the the hydrolysis of lactose in milk and whey, and products thereof.

Milk contains a carbohydrate, lactose, a disaccharide composed of the monosaccharides glucose and galactose. It is known that, due to a low intestinal lactase activity, not all humans are able to digest the lactose in milk with the same ease, resulting in lactose-intolerance. The production of lactose free milk and milk-derived products is a well-known application of the enzyme lactase. Lactase has been used for decades and the characteristics and use are described in many publications.

Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36, this use of an acid lactase from *Aspergillus oryzae* expressed in *Aspergillus niger* is

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Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36, this use of an acid lactase from *Aspergillus oryzae* expressed in *Aspergillus niger* is

exempt from premarket approval requirements of the Federal Food, Drug and Cosmetic Act, because the notifier has determined that such use is GRAS.


If you have any questions regarding this notification, or require any additional information to aid in the review of DSM's conclusion, please do not hesitate to contact me via email at gyingling@morganlewis.com or by telephone, (202)739-5610.

Sincerely,

(b) (6)

A large rectangular area of the document is redacted with a solid gray fill. To the left of this area, the text "(b) (6)" is written in red.

Gary L. Yingling

Handwritten signature of Gary L. Yingling in black ink, consisting of a stylized 'G' followed by 'ary L. Yingling'.

cc: DSM Food Specialties

**GRAS NOTIFICATION FOR ACID
LACTASE FROM *ASPERGILLUS ORYZAE*
EXPRESSED IN *ASPERGILLUS NIGER***

Submitted by:

DSM Food Specialties
PO Box 1
2600 MA Delft
The Netherlands

**GRAS NOTIFICATION FOR ACID LACTASE FROM *ASPERGILLUS ORYZAE*
EXPRESSED IN *ASPERGILLUS NIGER***

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1 GENERAL INTRODUCTION AND CLAIM OF EXEMPTION FROM PREMARKET APPROVAL REQUIREMENTS

DSM Food Specialties (“DSM”) manufactures the food enzyme acid lactase, which is produced by submerged fermentation of a selected, pure culture of a genetically modified organism (“GMO”) *Aspergillus niger* expressing the acid lactase gene from *Aspergillus oryzae*. DSM produces the acid lactase preparations in dried and liquid forms. The dried form is standardized with maltodextrin, while the liquid is standardized with glycerol. The trade name will be Maxilact® A4.

The described acid lactase preparation is intended for use in milk, whey and products thereof as a processing aid to hydrolyze lactose.

The treated milk, whey and products are used in a variety of food products for lactose-intolerant people.

Pursuant to the regulatory and scientific procedures set forth in the Proposed Rule “Substances Generally Recognized as Safe,” 62 Fed. Reg. 18937 (April 17, 1997) (proposed 21 C.F.R. § 170.36) (“GRAS Proposed Rule”), DSM has determined that its acid lactase enzyme from a GMO *Aspergillus niger* is a GRAS substance for the intended food applications and is therefore exempt from the requirement for premarket approval. Information on the enzyme and the production organism providing the basis for this GRAS determination is described in the following sections. General and specific information identifying and characterizing the enzyme, its applicable conditions for use, DSM’s basis for its GRAS determination and the availability of supporting information and reference materials for FDA’s review can be found in this Section.

The production organism, *Aspergillus niger*, has a long history of safe use and is discussed in Section 2. FDA has previously affirmed as GRAS several enzyme preparations from *Aspergillus niger*. See 21 C.F.R. §§ 184.1033 (Citric acid) and 184.1685 (Rennet and chymosin). In addition, FDA has subsequently received GRAS notifications for enzyme preparations, including several produced from genetically modified *Aspergillus niger* strains. Peroxidase, carboxypeptidase, lipase, asparaginase (2 notifications) and phospholipase A2 preparations from genetically modified *Aspergillus niger* strains derived from the same strain-lineage as the *Aspergillus niger* strain described in this dossier are examples of other such preparations to which FDA had no objections (see GRAS Notifications GRN 000402, GRN 000345, GRN 000296, GRN 000428 and GRN 000214 and GRN 000183, respectively). In a publication authored by FDA professionals, there was a summary of the safety of microorganisms, including *Aspergillus niger*, used as a host for enzyme-encoding genes (Olempska-Beer, Z.S. *et al.*, 2006).

Section 2 also describes the genetic modifications implemented in the development of the production microorganism to create a safe standard host strain resulting in a genetically well-characterized production strain, free from known harmful sequences. In Section 3, data showing acid lactase to be substantially equivalent to naturally occurring (acid) lactases are presented.

Section 4 discusses the safety of the materials used in the manufacturing process. Section 5 reviews the composition, specifications and general production controls. Section 6 provides

information on the mode of action, application, use levels and measurable enzyme residues in the final food products in which acid lactase is to be used. Finally, the safety studies outlined in Section 7 indicate that *Aspergillus niger* and acid lactase show no evidence of pathogenicity or toxicity. Estimates of human consumption and an evaluation of dietary exposure are also included in Section 7.

1.1 Name and Address of Notifier

NOTIFIER

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The Netherlands

MANUFACTURER

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1.2 Common or Usual Name of Substance

DSM's acid lactase enzyme preparation from *Aspergillus oryzae* expressed in *Aspergillus niger* is produced by submerged fermentation of a selected, pure culture of *Aspergillus niger*. The common or usual name of the substance is "acid lactase". Other names are lactase and beta-galactosidase. It is produced and sold in dried or liquid form. The dried form is standardized with maltodextrin; the liquid form is so with glycerol. The trade name will be Maxilact® A4.

1.3 Applicable Conditions of Use

Milk is a significant dietary source of at least eight nutrients, including protein and calcium (Phillips, M.C. and Briggs, G.M., 1975). Milk also contains a carbohydrate (lactose), a disaccharide composed of the monosaccharides glucose and galactose. It is known that, due to a low intestinal lactase activity, not all humans are able to digest the lactose in milk with the same ease. This so-called “lactose intolerance” is widespread within a substantial part of the world population (Modler, H.W. *et al.*, 1993). The percentage of lactose malabsorption in the Caucasian human race ranges from 2% (Danish) to approx. 19% (White American). However the indigenous populations of Eastern Europe, the Mediterranean, Africa, Asia, Latin America and Australia show a much higher incidence ranging from 60% (e.g. Indian, Israeli, Latin- and North-American Indians) to values of 80% (e.g. Chinese, Japanese, Nigerian, Australian aboriginal)

In the USA about one-third of the population, mainly African, Mexican and Asian Americans experience difficulty with the digestion of lactose or lactose containing food (Houts, S.S., 1988). These people cannot benefit from the nutritional quality of milk and milk derived products without having severe gastrointestinal (GI) complaints (cramps, flatulence, and diarrhea). Pre-treatment of the milk with acid lactase will prevent intestinal problems. In the USA, the National Medical Association (NMA¹) has in 1999 urged the Department of Agriculture to promote lactose free dairy foods in both the Food Guide Pyramid and Dietary Guidelines for Americans (US NMA, 1999). Milk and milk products are the most important source of calcium in the diet. Women who are lactose intolerant may be at greater risk of osteoporosis resulting from low Ca-intake.

The production of lactose free milk and milk-derived products is a well-known application of the enzyme lactase. Lactase has been used for decades and the characteristics and use are described in many publications (Dziezak, J.D., 1991, Holsinger, V.H. and Kligerman, A.E., 1991, Dahlqvist, A. *et al.*, 1977, Olling, C.C.J., 1972) and textbooks (Godfrey, T. and West, S., 1996, White, J.S. and White, D.C., 1997, Robinson, R.K., 1986, Nagodawithana, T.W. and Reed, G., 1993, Durand, G. and Monsan, P., 1982). Treatment of milk with lactase converts lactose into galactose and glucose. Persons with lactose intolerance can readily digest these monosaccharides without gastrointestinal problems. Treated milk can be used as such or further manufactured into ice cream, yoghurt or cheese.

Concentrated whey (protein) containing lactose is used as an ingredient in bakery, ice-creams and dressings. The low solubility of lactose (concentration problems), its sandiness and low sweetening power compared to glucose makes the hydrolysis of lactose attractive. Enzymatic hydrolysis is preferred over the acid hydrolysis of lactose because Maillard browning is prevented (Wigley, R.C., 1996).

¹ NMA is a national organisation of African American Physicians in the USA

In order for the enzyme to be able to perform a technological function in the final food, a number of conditions have to be fulfilled at the same time:

- the enzyme protein must be in its 'native' (non-denatured) form, AND
- the substrate must still be present, AND
- the enzyme must be free to move (able to reach the substrate), AND
- conditions like pH, temperature and water content must be favourable

In the below-mentioned applications there will either be a lack of substrate and/or the temperature or pH conditions will not be favorable. Therefore, the enzyme will not be active in the final food application and the acid lactase can thus be regarded as a processing aid in all applications.

1.3.1 Substances Used In

The acid lactase preparation is to be used in milk and whey and their products.

1.3.2 Levels of Use

Enzyme preparations are generally used in *quantum satis*. The average dosage of the enzyme depends on the application, the type and quality of the raw materials used, and the process conditions. Details on applications in milk and whey are given below.

Acid lactase applied in milk

As Thompson and Brower describe in 1976 (Thompson, M.P. and Brower, D.P., 1976), the amount of enzyme for the hydrolysis will depend on the degree of conversion desired and the time available. Conversions of 70% of the lactose can be obtained with 28 ALU/ml of milk held at 37°C for 6 hours. The same conversion can be obtained in one hour at 37°C, when the pH of the milk is lowered to pH 4.0.

If the digestion is carried on for periods longer than those indicated above, a smaller quantity of acid lactase will produce the same conversion.

Acid lactase applied in whey

The application dosage of acid lactase in whey is the same as used in milk.

1.3.3 Purposes

Acid lactase is proposed to be used for the hydrolysis of lactose in milk. The treated milk can then be used as:

Lactose-free Milk

As mentioned above, lactose-free milk can be drunk by lactose intolerant populations without giving the abdominal problems lactose containing (normal) milk does.

Dulce de leche, ice cream, cream

Lactose is hydrolysed by acid lactase in glucose and galactose. Due to the higher solubility of these single sugars, highly concentrated or frozen milk products can be obtained without the phenomenon of "sandiness." The latter is an advantage in the production of products like ice cream and dulce de leche. The level of hydrolysis needed to prevent crystallisation of lactose in dulce de leche is about 30%; higher levels can be used if increased sweetness is needed.

Yoghurt

Although most strains of starter organisms for yoghurt production, i.e. *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, have been selected for their ability to ferment lactose, the actual splitting of lactose appears to be the rate limiting step. The hydrolysis of the lactose therefore often further stimulates the growth of these cultures. In traditional fruit yoghurts or other yoghurt based desserts the lactose does not contribute to the sweetness. Glucose and galactose, being sweeter than lactose, substantially contribute to sweetness, thereby reducing the need for added sugar.

In addition, acid lactase can be used for the hydrolysis of lactose in whey (protein).

With a partial hydrolysis of the lactose, the sweetness of the end product and solubility of the sugars will be increased. The glucose/ galactose mixture has, depending on the concentration, a sweetness of 65-80% relative to sucrose. Microbiologically stable whey syrups up to 75% total solids can be prepared and can be used in the different application areas (Wigley, R.C., 1996),

Whey, whey protein concentrate and whey protein for human consumption

Whey (protein) as such is consumed by certain specific parts of the human population (e.g. bodybuilders).

Bakery Products: Bread, Biscuits and Cakes

Since whey syrup primarily consists of a mixture of non-denatured proteins and sugars, both chicken egg white and sucrose can be replaced. Up to 30% of the egg-white can be substituted, while the single sugars in the syrup contribute greatly to the improvement of the colour of the baked product.

Candies and Confectionary Products

Hydrolysed whey syrups can replace large percentages of the sweetened condensed milk used in the manufacture of toffees, fudge, candy bars and dulce de leche. No granulation or sandiness caused by lactose crystals will appear and the caramelization is improved.

Ice-cream, Frozen Yoghurt and Desserts

Hydrolysed whey can replace both sucrose and milk. The ice cream and frozen yoghurt will have better melting characteristics (lower freezing point). No sandiness will occur even during prolonged storage.

1.3.4 Consumer Population

Lactase hydrolyzes terminal non-reducing β -D-galactose residues in β -D-galactosides. Many living organisms (several kinds of bacteria, fungi, plants and animals, including mammals) possess lactases, which harmlessly and catalytically decompose terminal galactose residues to galactosides. As a result, lactase is abundant in the human diet. Hence, the addition of acid lactase in any of the proposed applications will have no significant effect on the human body. Acid lactase is an enzyme protein naturally occurring in microorganisms, animals and plants and the enzyme will be digested, as would any other protein occurring in food.

Lactases are abundantly present in nature as has been described in the BRENDA database². They can be found among mammals (human, dog and cow), plants (barley, carrot, rice, bean and pea), fungi (*Aspergillus* sp. and *Saccharomyces*) and bacteria (*Lactobacilli*, *Streptococcus thermophilus* and *Flavobacterium*) (Lau, H., 1987, Hotamisligil, S. *et al.*, 1993, Distler, J.J. and Jourdian, G.W., 1978, Hemavathi, A. *et al.*, 2008, Konno, H. *et al.*, 1988, Kaneko, S. and Kobayashi, H., 2003, Biswas, S. *et al.*, 2003, Dwevedi, A. and Kayastha, A.M., Watanabe, Y. *et al.*, 1979, Widmer, F. and Leuba, J.-., 1979, Li, W.Z. *et al.*, 2009, Kim, J.-. and Rajagopal, S.N., 2000, Honda, H. *et al.*, 2007, Park, A. and Oh, D., 2010, Smart, J. and Richardson, B., 1987, Sørensen, H. *et al.*, 2006). The addition of the acid lactase preparation to milk or whey will result in a reaction product equal to those formed in people who are lactose-tolerant.

As is shown in Section 6.4 of this dossier, the total amount of enzyme TOS in the final food is expected to be about 0.14 g/l milk or whey product (=0.014%).

Since acid lactase is present in food products at such low levels, and because it is a naturally occurring enzyme in both human cells and tissues and commonly ingested by humans, the consumer population will not be affected by the presence of the acid lactase preparation in food.

² <http://www.brenda-enzymes.org/>

1.4 Basis for GRAS Determination

Pursuant to the GRAS Proposed Rule, DSM has determined, through scientific procedures, that its acid lactase enzyme preparation from *Aspergillus oryzae* expressed in *Aspergillus niger* is GRAS for hydrolysis of lactose in milk, whey and products thereof, in levels not to exceed good manufacturing practices.

1.5 Availability of Information for FDA Review

The data and information that are the basis for DSM's GRAS determination are available for the FDA's review, and copies will be sent to FDA upon request. Requests for copies and arrangements for review of materials cited herein may be directed to:

Gary L. Yingling, Esq.
Morgan, Lewis & Bockius LLP
1111 Pennsylvania Avenue, NW
Washington, DC 20004-2541

2 PRODUCTION MICROORGANISM

2.1 Donor, Recipient Organism and Production Strain

Donor:

The gene coding for the acid lactase (*TOL* gene) was a codon optimized cDNA sequence from *Aspergillus oryzae*, *in vitro* synthesized.

Recipient organism

The recipient organism used in the construction of the acid lactase production strain is a glucoamylase (also called amyloglucosidase), protease, and amylase negative *Aspergillus niger* strain designated ISO-528 and stored in the DSM Culture Collection as DS 30829. The strain ISO-528 was declared as a suitable host strain for the construction of genetically modified organisms belonging to Group I safe microorganisms by the Dutch authorities (DSM relies on a letter from Director-General Milieu to DSM's Strain Director, dated 06-07-2005, included in Annex 2.1.1).

The strain ISO-528 is derived from the fully characterized DSM *Aspergillus niger* strain GAM-53 (DS 03043) by genetic modification. The strain GAM-53 was derived by several classical mutagenesis steps from *Aspergillus niger* strain NRRL 3122, a strain purchased from the Culture Collection Unit of the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois, USA.

The fully characterized strain *Aspergillus niger* GAM-53 was isolated by DSM (then: Gist-brocades) in 1982 and selected for its enhanced production of the endogenous enzyme glucoamylase. Since that time, strains of the GAM-lineage have been used at DSM for the large-scale production of glucoamylase, an enzyme that is utilized worldwide in the starch processing industry.

The strain GAM-53 was taxonomically identified as *Aspergillus niger* by the Dutch culture collection, the Centraalbureau voor Schimmelcultures (CBS) (DSM relies on a letter from CBS to Gist-Brocades, dated 29.1.1994 regarding Identification Service, Annex 2.1.2). This is an independent, internationally recognized laboratory.

The strain GAM-53 is being used to construct a new generation of strains according to the 'design and build' concept, in which introduced genes are targeted ('plugged') to a predetermined region of the genome. The exact technique used to construct such 'plug bugs' (designated as 'ISO-strains') and its advantages are described in literature (Selten, G.C.M. *et al.*, 1995, Van Dijck, P.W.M. *et al.*, 2003), included as Annex 2.1.3). ISO-strains were used for the construction of production strains for phospholipase A2 (donor: porcine pancreas, see GRAS notification GRN 000183), asparaginase (donor: *Aspergillus niger*, see GRAS notification GRN 000214 and GRN000428), lipase (donor: synthetic gene, see GRAS notification GRN 000296), carboxypeptidase (donor: *Aspergillus niger*, see GRAS notification GRN 000345), peroxidase (donor: *Marasmius scorodonius*, see GRAS notification GRN 000402), arabinofuranosidase

(donor: *Aspergillus niger*), phytase (donor: *Aspergillus niger*), pectin methyl esterase (donor: *Aspergillus niger*), glucoamylase (donor: *Aspergillus niger*), xylanase (donor: *Aspergillus niger*), endo-polygalacturonase (donor: *Aspergillus niger*), proline specific endo-protease (donor: *Aspergillus niger*) and amylase (donor: *Aspergillus niger*).

The recipient organism ISO-528 used in the construction of the acid lactase production strain was derived from GAM-53 as follows:

The strain GAM-53 contains 7 loci (i.e. the promoter and coding sequences) for the glucoamylase gene. These 7 loci were removed, creating so-called 'plug-sites' (also called *ΔglaA loci*) into which expression units containing various genes can be integrated ('plugged'). The 7 'plug-sites' were each provided with unique restriction sites (also called 'DNA-flags'), marking the location of the 'plug-sites' on the genome. In addition, the gene coding for the major protease (*pepA*) was inactivated and the major amylases (*amyA* and *amyB*) were deleted both by established rDNA techniques and the strains capacity to secrete proteins was improved by classical mutation and selection.

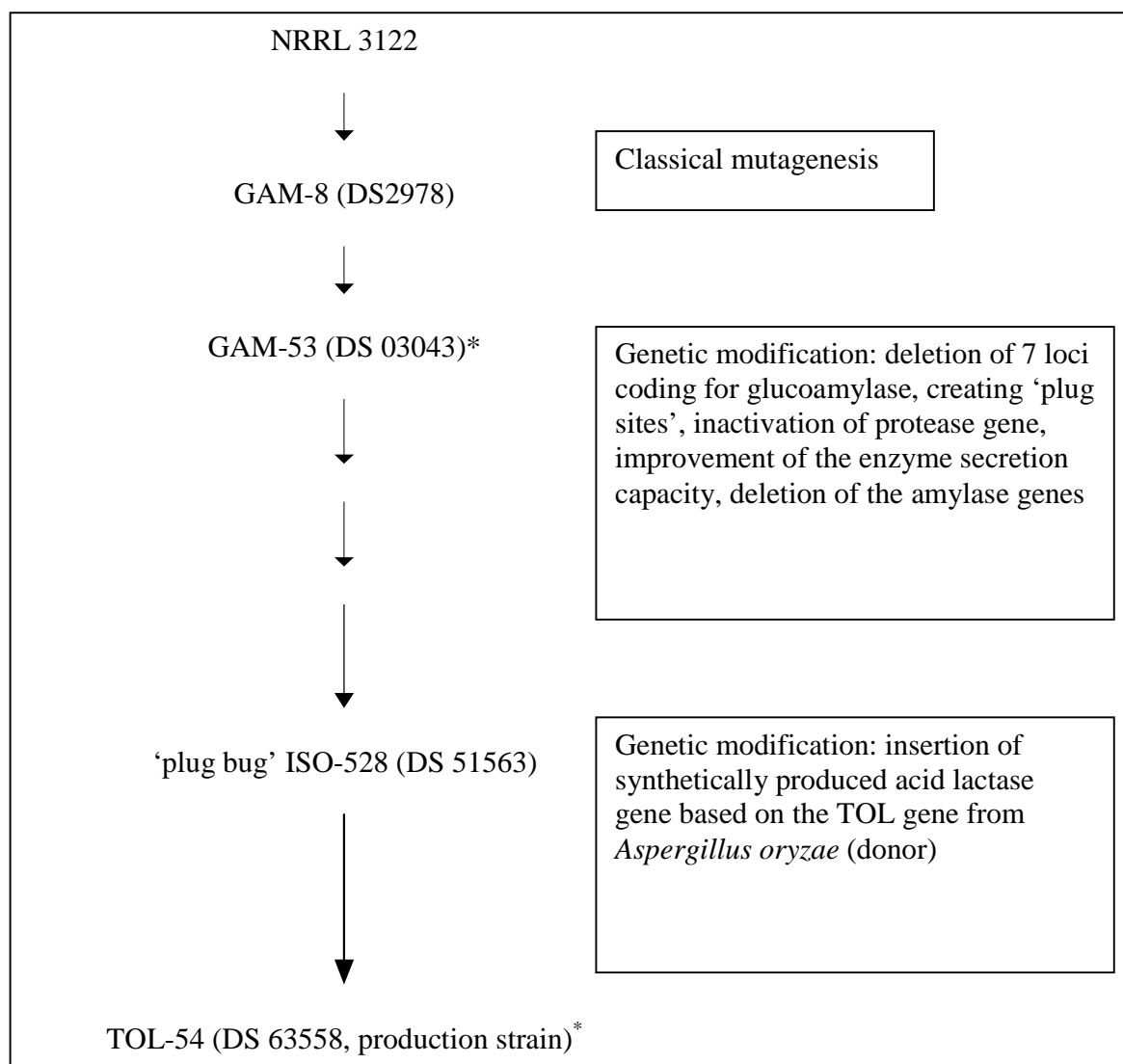
The resulting 'plug bug', ISO-528, was classified as a GMO by the Dutch competent authorities (DSM relies on a letter from Director-General Milieu to DSM's Strain Director, dated 06-07-2005, Annex 2.1.4).

The ISO-528 has been used for the construction of the product strains for asparaginase (donor: *Aspergillus niger*), lipase (synthetic gene), carboxypeptidase (donor: *Aspergillus niger*) and peroxidase (donor: *Marasmius scorodonius*). All four enzymes are subject to GRAS Notifications (GRN 000214, GRN 000296, GRN 000345 and GRN 000402). FDA had no questions regarding these GRAS notifications.

Production strain

The acid lactase production strain was obtained by further genetic modification of the *Aspergillus niger* strain ISO-528. The genetic modification techniques used are described in Section 2.2 of this dossier. The production strain was designated TOL-54 and stored in the DSM Culture Collection as DS63558.

Below, a schematic presentation of the genealogy of the production strain is given.



* Strain GAM-53 and TOL-54 were taxonomically identified as *Aspergillus niger* by the Dutch culture collection, the Centraalbureau voor Schimmelcultures (CBS).

As is shown in Section 2.4, the production strain complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms. It also meets the criteria for a safe production microorganism as described by Pariza and Johnson in 2001 (Pariza, M.W. and Johnson, E.A., 2001) and other expert groups (Berkowitz, D. and Maryanski, J., 1989, Commission of the European Communities, 1992, Organisation for Economic Co-operation and Development, 1993, Jonas, D.A. *et al.*, 1996, Battershill, J.M., 1993).

2.2 Genetic modification

For the construction of the acid lactase production strain, two plasmids were used: one to derive the expression cassette, containing the acid lactase gene, and the other to derive the cassette containing a selectable marker.

Donor DNA

The TOL gene is a codon-optimized cDNA coding sequence from *Aspergillus oryzae*, *in vitro* synthesized. It leads to an enzyme which is identical to the enzyme from *Aspergillus oryzae*.

Acid lactase expression plasmid

The obtained fragment was ligated in the pGBTOP vector resulting in the final pGBTOPTOL-1 plasmid and propagated in *E. coli*.

Summarizing, the vector pGBTOPTOL-1 comprising the acid lactase enzyme expression unit contains the following genetic elements:

- | | |
|-------------------|--|
| PglaA: | a 2.0 kB sized <i>glaA</i> promoter sequence of <i>Aspergillus niger</i> GAM-53, both for controlling the TOL expression and for targeting the expression unit to the Δ <i>glaA</i> loci. |
| TOL: | the entire 3018 bp sized codon optimized cDNA sequence encoding the acid lactase protein of <i>Aspergillus oryzae</i> from the ATG initiation codon to the TAA termination signal. The sequence of the acid lactase coding DNA results in a acid lactase pre(pro)enzyme of 1005 amino acids in size. |
| 3'- <i>glaA</i> : | the 2.2 kB sized 3' flanking <i>Aspergillus niger</i> host-own <i>glaA</i> sequence for efficient termination of acid lactase (TOL) gene transcription and targeting of the expression unit to the Δ <i>glaA</i> loci. |
| pTZ18R: | see Section 2.4. |

The TOL expression unit is defined as the functional part of expression vector pGBTOPTOL-1. The TOL expression unit comprises the TOL gene, the expression of which is placed under control of the PglaA promoter.

Selectable marker plasmid

The selectable marker plasmid contains the same defined parts of the *Aspergillus niger* glucoamylase locus as the expression plasmid, the promoter sequence of the glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) gene from the *Aspergillus niger* related fungus *Aspergillus nidulans*, the *Aspergillus nidulans amdS* (acetamidase) selectable marker gene and DNA from a well-characterized *Escherichia coli* vector pTZ18R.

The different elements of the plasmid are:

- The *glaA* promoter (PglaA) from the parental *Aspergillus niger* strain GAM-53.
- The *gdpA* promoter (PgdpA) from *Aspergillus nidulans*.
- The *amdS* gene from *Aspergillus nidulans*.

- A 3'-flanking *glaA* terminator sequence from the parental *Aspergillus niger* strain GAM-53.
- DNA sequences from the *E. coli* plasmid pTZ18R. These sequences are removed prior to transformation of the *amdS* selection cassette into the host.

Transformation and selection of the final production strain

The TOL and *amdS* expression units – both completely free of any *E. coli* DNA – were integrated into the genome of the host ISO-528 by co-transformation. Due to the homology in the 3'-*glaA* and P*glaA* parts of the two expression units, they are preferentially targeted to one of the seven Δ *glaA* loci, the *Bam*HI Δ *glaA* locus.

The transformants are selected on agar plates containing acetamide as the sole carbon source. By further analysis transformants are selected that have multiple copies of the acid lactase expression cassette and one or more copies of the selection marker cassette integrated into one of the Δ *glaA* loci of the recipient strain. The selection of these transformants was done by PCR analyses, applying TOL and 3'-*glaA* specific primers.

By counter-selection on fluoro-acetamide containing plates, a natural variant was selected in which the *amdS* selection markers were deleted as a result of a natural internal recombination event. The absence of the *amdS* marker was confirmed by Southern analysis. The resulting organism is thus not only totally free of *E. coli* DNA, but also of the *amdS* selection marker. This strain was designated TOL528-8 (DS62369) and was shown to contain 6 TOL gene copies. Starting with such a natural variant it is possible to multiply the region comprising the expression unit(s) and the “DNA-flag” into the other Δ *glaA* loci by so-called “gene conversion” (Selten, G.C.M. *et al.*, 1998), a natural spontaneous recombination event which does not involve mutagenic treatment. Strains that have an increase in the copy number of the “DNA flag” marking the filled Δ *glaA* locus and a consequent loss of the other “DNA-flags” can easily be identified by DNA gel electrophoresis.

From the available recombinants a strain was chosen that contained 24 TOL gene copies which was designated as TOL528-17 (DS63557). After further testing to check the production levels, the strain was mass cultured and renamed TOL-54 (DS63558).

The expression unit TOL is translated into an acid lactase protein, which is glycosylated and secreted into the medium as a mature, active enzyme.

2.3 Stability of the Transformed Genetic Sequence

The strains belonging to the *Aspergillus niger* GAM-lineage - from which both the host ISO-528 and the present recombinant acid lactase production strain TOL-54 are derived - are genetically stable strains. The whole GAM-lineage is stored for more than 30 years at the DSM laboratory. New cultures are frequently derived from stock material and tested after many generations on morphological, growth, production, and product characteristics. These characteristics remain stable except that after plating out, a low frequency of morphologic dissimilar colonies are found. This, however, is a normal phenomenon observed for the parental as well as the highly

selected industrial strains. The stability of production strains from this ISO strain lineage, such as the acid lactase production strain, in terms of behavior in strain management and in terms of enzyme production characteristics, does not differ from the parental GAM-strains or from production strains constructed by random integration.

Since the acid lactase expression unit is integrated into the genome and since the expression unit does not contain an *E. coli* origin for replication (all *E. coli* sequences have been removed from the vector prior to transformation, see Section 2.5), it is not possible that the expression unit will be transferred from the *Aspergillus niger* production organism to another, non-related, organism.

2.4 Good Industrial Large Scale Practice (GILSP)

The acid lactase production organism complies with all criteria for a genetically modified GILSP organism.

The host organism is non-pathogenic, does not produce adventitious agents and has an extended history of safe industrial use (see Section 7.1). The ancestor of the host, GAM-53 (see Section 2.1) has been shown to have limited survival outside the optimal conditions of the industrial fermentor (see Annex 2.4.1). Given the nature of the genetic modifications performed, there are no reasons to believe that the survival of the genetically modified production organism would be different when compared to its ancestor. The DNA insert is fully characterized and is free from known, harmful sequences. No antibiotic resistance markers or other heterologous markers are present in the strain.

Therefore, consistent with the principles of Good Industrial Large Scale Practice (GILSP), as endorsed by the Organization of Economic Cooperation and Development (OECD), the acid lactase production organism is considered to be of low risk and can be produced with minimal controls and containment procedures in large-scale production. The production organism has been approved both by the Dutch (DSM relies on a letter from Director-General Milieu to DSM Strain Director, dated 1 December 2009 in Annex 2.4.2)) and French competent authorities (DSM relies on a letter from the Comité Scientifique du Haut Conseil des Biotechnologies dated 2 March 2010 in Annex 2.4.3) for large-scale productions, under containment conditions not exceeding the GILSP level of physical containment. In the facilities of DSM Food Specialties, fermentations for the large-scale production of food and feed enzyme products are carried out below the GILSP level of physical containment.

2.5 Absence of transferable rDNA Sequences in the Enzyme Preparation

As explained above, the expression unit contains no *Escherichia coli* origin for replication. As a result, the enzyme preparation will not contain any transferable rDNA sequences. In accordance with the rational design of the recombinant production strain, i.e., absence of any *Escherichia coli* plasmid or marker gene DNA, no transformable rDNA could be detected in the product by test.

2.6 Absence of Production Organism in the Product

In accordance with the recommendations for safety evaluation by the International Food Biotechnology Committee (Coulston, F. and Kolbye, A.C., 1990b), all traces of the production organism are removed during the manufacturing process (see Section 4.4), ensuring that the enzyme preparations are free from the production organism *Aspergillus niger*.

2.7 Absence of Antibiotic Resistance Gene

As noted above, no antibiotic resistance markers or other heterologous markers are present in the strain. The enzyme preparations are tested to ensure the absence of antibiotic activity in accordance with the recommendation from the Joint Expert Committee of Food Additives of the FAO/WHO ("JECFA"). As is shown in Section 4.6 of this dossier, quality control testing of the finished acid lactase preparations ensures the enzymes do not contain antibiotic activity.

2.8 Absence of Toxins

The Food Chemicals Codex ("FCC", 8th edition), states the following: *"Although limits have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants."*

In the General Specifications for enzyme preparations laid down by JECFA in 2006, the following is said: *"Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis. Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species."*

DSM devised a test protocol to determine if the acid lactase-producing microorganism possesses the intrinsic capacity to produce mycotoxins. This was tested under routine conditions of industrial submerged fermentation. The test showed that the production strain does not produce any known toxins under the routine conditions of industrial submerged fermentations, and therefore no toxins are expected in the final enzyme product.

3 ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

Systematic name	: β -D-galactoside galactohydrolase
Accepted name	: β -galactosidase
Other names	: Acid lactase; lactase; β -lactosidase; maxilact; hydrolact; β -D-lactosidase; S 2107; lactozym; trilactase; β -D-galactanase; oryzatym; sumiklat
Enzyme Commission No.	: 3.2.1.23

Acid lactase belongs to the subclass of glycosidases.

3.2 Amino Acid Sequence

The acid lactase described in this dossier is produced by *Aspergillus niger* as a homo-dimeric glycoprotein with a primary sequence of 987 amino acids. Based on SDS-PAGE, it can be concluded that the molecular weight of the enzyme is about 97 kDa after secretion. Annex 3.2.1 shows the amino acid sequence of the acid lactase produced by a GMO *Aspergillus niger*.

3.3 Sequence Comparison to Other Acid lactases

In order to compare the acid lactase produced by a GMO *Aspergillus niger* with other comparable enzymes, an extensive sequence comparison study has been carried out. The amino acid sequence of the *Aspergillus niger* acid lactase was used as a query to search sequence databases using 'BLAST' software for related sequences.

For 14 organisms, enzyme sequences with significant ($E\text{-value} \leq 5 \times 10^{-38}$)³ homology were found.

The most similar sequences are beta-galactosidases (also called lactase, see <http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/2/1/23.html>) from related Ascomycetes. They have E-values below 1×10^{-104} . Ascomycetes are in general so-called 'sac-fungi', a group which is of particular relevance to humans as source for medicinally important compounds, such as antibiotics and for making bread, alcoholic beverages, and cheese, but also as pathogens of humans and plants. Familiar examples of sac fungi include morels, truffles, brewer's yeast and baker's yeast, Dead Man's Fingers, and cup fungi. Also *Aspergillus* species are part of this group.

³ E-values indicate the degree of similarity in sequences, the lower the E-value, the more similar the sequence. An E-value of 0 indicates identical sequences.

Finally, other sequences with a more distant homology with BLASTp E-values between 1×10^{-22} and 1×10^{-37} are from plants and mammals. Among those species are rice, soy, corn and pear for the plants and pig, dog, elephant and horse for the mammals.

3.4 Enzymatic Activity

Principal Enzyme Activity

The enzyme, acid lactase, hydrolyzes terminal non-reducing β -D-galactose residues in β -D-galactosides, like for example in lactose. This milk sugar is hydrolyzed into β -D-galactose and α -D-glucose. The activity of acid lactase in Maxilact® A4 is expressed in so-called Acid lactase Units (ALU).

One ALU is defined as that quantity of enzyme that will liberate o-nitrophenol (ONP) at a rate of 1 μ mol per minute under the conditions of the assay.

This assay method for acid lactase is published in the Food Chemical Codex 8th ed (Food Chemical Codex, 2012). The method is based on the release of the o-nitrophenyl group from the synthetic substrate O-NitroPhenyl- β -D-Galactopyranoside (ONPG) at pH 4.5 and 37°C. The formed (yellow) o-nitrophenol, which is determined in a suitable spectrophotometer at 420 nm in a 1-cm cell, is a measure for the acid lactase activity.

The molecular weight (MW) of the enzyme from *Aspergillus oryzae* and produced in *Aspergillus niger*, deduced from the amino acid sequence, is 107 kDa. This is of the same order as other known acid lactases (*Aspergillus niger*, 109 kDa, *Kluyveromyces lactis* 118 kDa and *Aspergillus oryzae* itself, at 107 kDa).

The enzyme activity has an pH optimum between 4.0 – 4.5, and has more than 80 % activity between pH 3 – 5. Hence, this is where the name acid lactase comes from.

The temperature optimum of this acid lactase lies around 55°C, and the enzyme is more than 80% active between 40-60°C (Fullbrook, P.D., 1996).

The enzyme is stable (1 hour 37°C, $\geq 80\%$ residual activity) in the pH range 3-8, see figure 1.2, but starts to inactivate at temperatures above 55°C at a pH of 4.5-5.0.

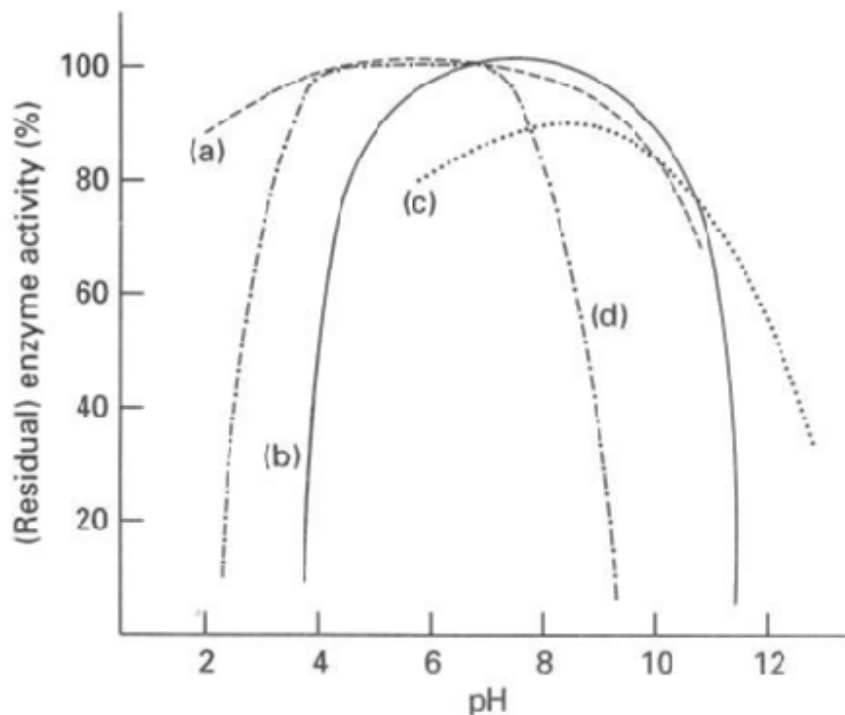


Figure 1.2, pH stability curve for four industrial enzymes. (a): *Aspergillus niger* alpha-amylase; (b): *Bacillus licheniformis* alkaline protease; (c): an enzyme from the same strain; and (d) *Aspergillus oryzae* beta-galactosidase, after 1 hour at 37°C (Fullbrook, P.D., 1996).

Subsidiary enzymatic activities

Like any other living micro-organism, the acid lactase production organism *Aspergillus niger* produces many other enzymes needed for the breakdown of nutrients and buildup of cell material.

Although acid lactase is being produced in excess, the enzyme preparation will also contain minor, non-standardized amounts of these other enzymes. These amounts do not have an effect (positive or negative) in the applications.

4 MANUFACTURING PROCESS

4.1 Overview

The acid lactase described in this dossier is produced by a controlled submerged fermentation of a selected, pure culture of a GMO *Aspergillus niger* (see Section 2). The production process includes the fermentation process, recovery (downstream processing) and formulation of the product. An overview of the different steps involved is given in Annex 4.1.1.

4.2 Raw Materials

The raw materials used for the fermentation and recovery of the product are suited for the intended use leading to the required safety status of the product. This is confirmed by the toxicological studies performed (see Section 7.4 of this dossier). The raw materials meet predefined quality standards that are controlled by the Quality Assurance Department of DSM. The raw materials used for the formulation are of food grade quality.

The fermentation medium used has been developed for optimum production of enzymes (in this case acid lactase) by the DSM *Aspergillus niger* hosts.

4.3 Fermentation Process

The acid lactase is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *Aspergillus niger* described in Section 2. All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and confirm strain identity.

The fermentation process consists of three steps: pre-culture fermentation, seed fermentation and main fermentation. The whole process is performed in accordance with current Good Manufacturing Practices.

Biosynthesis and excretion of acid lactase occur during the main fermentation. To produce the enzyme of interest, a carefully controlled, submerged, aerobic fed batch fermentation process is employed under aseptic conditions, using a stirred tank fermentor.

Growth of the production organism and increase of enzyme production are checked at the end of the main fermentation by analysis of aseptically collected samples. After the fermentation is stopped, downstream processing begins.

4.4 Recovery Process

The fermentation is stopped by addition of sodium benzoate under conditions that effectively kill off the production organism.

The cell material is separated from the enzyme by means of a simple solid-liquid filtration process. Subsequently, the remaining particles are removed with a polish filtration and a germ reduction filtration, and then concentrated by ultrafiltration (UF) and diafiltration (DF).

4.5 Stabilization, Formulation and Standardization Process

After ultrafiltration, the purified acid lactase (eluate) is again filtered with a polish and a germ reduction filtration and finally, it is formulated to a either a liquid or a dry preparation.

The liquid product is stabilized with glycerol to reach an acid lactase activity of ≥ 5000 ALU/g, in accordance with the product specification. Finally the product is filter sterilized.

The dry product is made by spray drying and granulation of the concentrate with maltodextrin. Finally it is standardized to the desired acid lactase activity of > 100.000 ALU/g with the same filling agent.

4.6 Quality Control of Finished Product

In accordance with the general specifications for enzyme preparations used in food processing as established by the Joint Expert Committee of Food Additives (JECFA) of the FAO/WHO in 2006 and the FCC (8th edition), the final acid lactase preparation from a GMO *Aspergillus niger* meets the following specifications:

<u>ITEM</u>	<u>NORM</u>
Lead	≤ 5 mg/kg
Coliforms	≤ 30 /g
<i>Salmonella</i>	0/25
<i>Escherichia coli</i>	0/25 g
Antimicrobial activity	Absent by test
Mycotoxins	Absent by test

The additional characteristics for the liquid formulation are:

<u>ITEM</u>	<u>NORM</u>
Acid lactase activity	≥ 5000 ALU/g
Appearance	Very light brown to brown
Glycerol	$> 50\%$
pH	4.3 – 4.7

The additional characteristics for the dry formulation are:

<u>ITEM</u>	<u>NORM</u>
Acid lactase activity	> 100.000 ALU/g
Appearance	White to off white granulates
Dry matter	> 94%

5 COMPOSITION AND SPECIFICATIONS

5.1 Formulation

The common starting material for all formulations is the UF concentrate. Typically, its composition falls within the following ranges:

Item	Value	Unit
Enzyme activity	25000-35000	DBLU/g
Water	80 – 90	%
Ash	0 – 0.5	%
Total Organic Solids	12.5 – 20	%

Apart from the enzyme complex, the acid lactase preparations will also contain some substances derived from the microorganism and the fermentation medium. These harmless contaminations consist of polypeptides, proteins, carbohydrates and salts.

The Total Organic Solids (“TOS”) of the acid lactase preparations were calculated from 3 different batches of the UF concentrate:

Calculation of the TOS					
Batch	Dry matter (% w/w)	Ash (% w/w)	TOS (% w/w)	Activity (ALU/g)	ALU/mg TOS
1	17.2	0.148	17.1	25900	151.5
2	15.8	0.0916	15.7	31650	201.6
3	16.4	0.18	16.2	29750	183.6
MEAN					178.9

Based on the above figures it can be calculated that the formulated commercial products with activities of 100.000 ALU/g will have a TOS value between 1.5 and 2.0 mg/g enzyme preparation.

5.2 General Production Controls and Specifications (Good Manufacturing Practice)

Commercial demands require a strictly controlled fermentation process.

The enzyme fermentation factory at Seclin, France, which has fermentation experience since 1922, has acquired the ISO 9001-2000 certification.

Technical measures

The batches of primary seed material are prepared, preserved and stored in such a way that contamination and degeneration is avoided and genetic stability is secured. The vials are clearly labeled and strict aseptic techniques are applied during the recovery of the culture.

Only sterilized raw materials are used to prepare the nutrient medium for the fermentation.

The fermentor is a contained system. Only sterilized air is used in the fermentation. Membrane valves, air filters and seals are regularly checked, cleaned and replaced if necessary. Prior to inoculation, the fermentor is cleaned, rinsed and sterilized. The sterilized nutrient medium and the complete biomass broth are transferred aseptically to the main fermentor. The methods used effectively prevent microbial contamination during fermentation.

The preparation of sterile media and the cleaning of the equipment are laid down in Quality Assurance documents and strictly followed.

Microbial contamination is prevented during downstream processing by several germ reduction filtrations. The filters are thoroughly cleaned for each production run.

Control measures

After preparation of a new batch of primary seed material, samples are checked for identity, viability and microbial purity. If these parameters are correct, the strain is tested for production capacity. Only if the productivity and the product quality meet the required standards, the new batch of primary seed material will be accepted for further production runs. Each time a vial from such a certified batch of primary seed material is used for production, the viability, purity and identity of the strain is checked.

The raw materials used for the fermentation and recovery of the product are suited for the intended use leading to the required safety status of the product. The raw materials meet predefined quality standards that are controlled by the Quality Assurance Department of DSM. The raw materials used for the formulation are of food grade quality.

At regular intervals during the seed fermentation manual samples are taken aseptically for analysis of pH, and microbiological quality in the laboratory.

During the main fermentation the dissolved oxygen content, pH, temperature, viscosity and microbial quality are monitored. If microbial controls show that significant contamination has occurred, the fermentation will be discontinued.

Also during downstream processing samples are being taken and checked for the level of microbial contamination. If these checks show that significant contamination has occurred, the downstream processing will be discontinued.

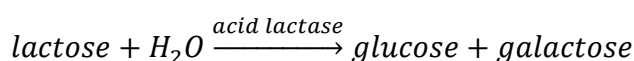
The finished product is subjected to extensive controls and complies with JECFA and Food Chemical Codex specifications. See Section 4.6: Quality Control of Finished Product.

6 APPLICATION

6.1 Mode of Action

Lactose is a disaccharide composed of the monosaccharides glucose and galactose, which is not well digested by certain populations.

Acid lactase can hydrolyze lactose in milk, whey and products of either. It hydrolyses the terminal non-reducing β -D-galactose residues in β -D-galactosides. In this specific case, it catalyzes the following reaction:



6.2 Application

The acid lactase in question is to be used in milk, whey and products of either.

The production of lactose free milk and milk-derived products is a well-known application of the enzyme lactase. Lactase has been used for decades and the characteristics and use are described in many publications (Dziezak, J.D., 1991, Holsinger, V.H. and Kligerman, A.E., 1991, Dahlqvist, A. *et al.*, 1977, Olling, C.C.J., 1972) and textbooks (Godfrey, T. and West, S., 1996, White, J.S. and White, D.C., 1997, Robinson, R.K., 1986, Nagodawithana, T.W. and Reed, G., 1993, Durand, G. and Monsan, P., 1982)Milk

Treatment of milk with lactase converts lactose into galactose and glucose. Persons with lactose intolerance can readily digest these monosaccharides without gastrointestinal problems.

- Lactose-free milk

Milk is a significant dietary source of at least eight nutrients, including protein and calcium (Phillips, M.C. and Briggs, G.M., 1975).

Milk also contains one carbohydrate (lactose), which is not well digested by certain populations. Lactose intolerant people cannot benefit from the nutritional quality of milk and milk derived products without having severe gastrointestinal (GI) complaints (cramps, flatulence, and diarrhea). This so-called “lactose intolerance” is widespread within a substantial part of the world population (Modler, H.W. *et al.*, 1993). In the USA for example about one-third of the population, mainly African, Mexican and Asian Americans experience difficulty with the digestion of lactose or lactose containing food (Modler, H.W. *et al.*, 1993, Houts, S.S., 1988).

In the USA, the National Medical Association (NMA⁴) has urged the Department of Agriculture to promote lactose free dairy foods in both the Food Guide Pyramid and Dietary Guidelines for Americans (US NMA, 1999).

Milk and milk products are the most important source of calcium in the diet. Women who are lactose intolerant may be at greater risk of osteoporosis resulting from low calcium-intake.

- Dulce de leche, ice cream, cream

Due to the higher solubility of the single sugars, highly concentrated or frozen milk products can be obtained without the phenomenon of "sandiness." The latter is an advantage in the production of products like ice cream and dulce de leche. The level of hydrolysis needed to prevent crystallisation of lactose in dulce de leche is about 30%; higher levels can be used if increased sweetness is also needed.

- Yoghurt

Although most strains of starter organisms for yoghurt production, i.e. *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, have been selected for their ability to ferment lactose, the actual splitting of lactose appears to be the rate limiting step. The hydrolysis of the lactose therefore often further stimulates the growth of these cultures. In traditional fruit yoghurts or other yoghurt based desserts, the lactose does not contribute to the sweetness. Hydrolysed lactose, being 3 times sweeter, substantially contributes to sweetness, thereby reducing the need for added sugar.

In addition, acid lactase can be used for the hydrolysis of lactose in whey (protein).

Concentrated whey (protein) is used as an ingredient in bakery, ice-creams and dressings. The low solubility of lactose (concentration problems), its sandiness and low sweetening power compared to glucose makes the hydrolysis of lactose attractive. Enzymatic hydrolysis is preferred over the acid hydrolysis of lactose because Maillard browning is prevented (Wigley, R.C., 1996).

With a partial hydrolysis of the lactose, the sweetness of the end product and solubility of the sugars will be increased. The glucose/galactose mixture has, depending on the concentration, a sweetness of 65-80% relative to sucrose. Microbiologically stable whey syrups up to 75% total solids can be prepared and can be used in the different application areas, Wigley R.C. 1996.

The hydrolyzed whey can then be used in the following applications:

⁴ NMA is a national organisation of African American Physicians in the USA

- Whey, whey protein concentrate and whey protein for human consumption

Whey (protein) as such is consumed by certain specific parts of the human population (e.g. bodybuilders).

- Bakery Products: Bread, Biscuits and Cakes

Since whey syrup primarily consists of a mixture of undenatured proteins and sugars, both chicken egg white and sucrose can be replaced. Reports indicate that up to 30% of the egg-white can be substituted, while the single sugars in the syrup contribute greatly to the improvement of the colour of the baked product.

- Candies and Confectionary Products

Hydrolysed whey syrups can replace large percentages of the sweetened condensed milk used in the manufacture of toffees, fudge, candy bars and dulce de leche. No granulation or sandiness caused by lactose crystals will appear and the caramelization is improved.

- Ice-cream, Frozen Yoghurt and Desserts

Hydrolysed whey can replace both sucrose and milk. The ice cream and frozen yoghurt will have better melting characteristics (lower freezing point). No sandiness will occur even during prolonged storage.

6.3 Use Levels

Enzyme preparations are generally used in *quantum satis*. The average dosage of the enzyme depends on the application, the type and quality of the raw materials used, and the process conditions. Details on applications in milk and whey are given below.

Acid lactase applied in milk

As Thompson and Brower (1976) describe, the amount of enzyme for the hydrolysis will depend on the degree of conversion desired and the time available. Conversions of 70% of the lactose can be obtained with 28 ALU/ml of milk held at 37°C for 6 hours. The same conversion can be obtained in one hour at 37°C, when the pH of the milk is lowered to pH 4.0.

If the digestion is carried on for periods longer than those above indicated, a smaller quantity of acid lactase will produce the same conversion.

Acid lactase applied in whey

The application dosage of acid lactase in whey is the same as used in milk.

6.4 Enzyme Residues in the Final Food

6.4.1 Residues of inactive enzyme

Based on the information given in Sections 5.1 and 6.3, and assuming that all the TOS of the lactase preparation ends up in the final food product, the following calculation can be made:

Final food	Enzyme use level in food ingredient	Amount of ingredient in final food	Residual amount of (inactivated) enzyme in final food	Amount of TOS in final food
Milk based products	28 ALU/ml milk	up to 100%	28 ALU/ml milk product	0.157 g TOS/l milk or milk product
Whey based products	28 ALU/ml whey	up to 80%	22.4 ALU/ml whey product	0.125 g TOS/l whey product

Fate of acid lactase in the end products

In order for the enzyme to be able to perform a technological function in the final food, a number of conditions have to be fulfilled at the same time:

- the enzyme protein must be in its ‘native’ (non-denatured) form, AND
- the substrate must still be present, AND
- the enzyme must be free to move (able to reach the substrate), AND
- conditions like pH, temperature and water content must be favourable

As there will be a lack of substrate and/or temperature or pH conditions are not favorable in the applications, the enzyme will not be active in the final food application and serve as a normal food protein.

6.4.2 Possible Effects on Nutrients

Lactase activity is widespread in nature. As can be seen in the BRENDA enzyme database, it can be found among mammals (human, dog and cow), plants (barley, carrot, rice, bean and pea), fungi (*Aspergillus* sp. and *Saccharomyces*) and bacteria (*Lactobacilli*, *Streptococcus thermophilus* and *Flavobacterium*) (Lau, H., 1987, Hotamisligil, S. *et al.*, 1993, Distler, J.J. and Jourdian, G.W., 1978, Hemavathi, A. *et al.*, 2008, Konno, H. *et al.*, 1988, Kaneko, S. and Kobayashi, H., 2003, Biswas, S. *et al.*, 2003, Dwevedi, A. and Kayastha, A.M., Watanabe, Y. *et al.*, 1979, Widmer, F. and Leuba, J.-., 1979, Li, W.Z. *et al.*, 2009, Kim, J.-. and Rajagopal, S.N., 2000, Honda, H. *et al.*, 2007, Park, A. and Oh, D., 2010, Smart, J. and Richardson, B., 1987, Sørensen, H. *et al.*, 2006).

The enzyme acid lactase is a protein and will be digested like all other proteins in the gastrointestinal system of humans.

The reaction products galactose and glucose are common monosaccharides, which are normal constituents of a diet. Moreover, the enzymatic degradation of lactose, from non-treated milk, takes place (by endogenous acid lactase) in the gastro-intestinal system of humans that do not suffer from lactose intolerance and results in the same reaction products.

The nutritional value of milk, whey and derived products is not changed or impaired by the application of acid lactase from *Aspergillus niger*.

7 SAFETY EVALUATION

7.1 Safety of the Donor and Production Strain

The safety of the production organism is paramount to assessing the probable degree of safety for enzyme preparations to be used in food production. According to the International Food Biotechnology Council (“IFBC”), food or food ingredients are safe to consume if they have been produced, according to current Good Manufacturing Practices, from a nontoxigenic and nonpathogenic organism (Coulston, F. and Kolbye, A.C., 1990a). A nontoxigenic organism is defined as “one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure” and a nonpathogenic organism as “one that is very unlikely to produce disease under ordinary circumstances” (Pariza, M.W. and Foster, E.M., 1983).

Aspergillus niger is known to naturally occur in foods. The fungus is commonly present in products like rice, seeds, nuts, olives and dried fruits.

For several decades, *Aspergillus niger* has been safely used in the commercial production of organic acids and various food enzymes, such as glucose oxidase, pectinase, alpha-amylase and glucoamylase. Industrial production of citric acid by *Aspergillus niger* has taken place since 1919 (Schuster, E. *et al.*, 2002) attached as Annex 7.1.1.

This long experience of industrial use has resulted in a good knowledge of the characteristics of *Aspergillus niger* and understanding of the metabolic reactions.

The long industrial use and wide distribution of *Aspergillus niger* in nature has never led to any pathogenic symptoms. The nonpathogenic nature has been confirmed by several experimental studies (see Annex 7.1.1). *Aspergillus niger* is therefore generally accepted as a nonpathogenic organism.

Even though products from *Aspergillus niger* have been used in food for many decades, there is no evidence that the industrial strains used produce toxins under the routine conditions of industrial submerged fermentations. The safety has been confirmed by a large amount of toxicological tests, as well as batch testing of the various end products for toxins.

The toxicological studies performed on various enzyme preparations from *Aspergillus niger* provided the basis for a safety evaluation by the Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO in 1988 (see Annex 7.1.2). Although not justified by the results of the toxicological studies, JECFA first allocated a numerical Acceptable Daily Intake (ADI) to enzyme preparations of *Aspergillus niger*, based on the concern that some strains may produce unknown toxins. Two expert reports submitted to JECFA in 1988 concluded that the production of toxins was highly unlikely (see Annex 7.1.3). The long history of use as an enzyme source, the numerous toxicological studies and the two expert reports caused JECFA to review its decision in 1990 and change the ADI for enzyme preparations derived from *Aspergillus niger* into “not specified”. See Annex 7.1.4 to this notification for JECFA’s 1990 review.

In addition to the positive evaluation of JECFA, countries which regulate the use of enzymes, such as the USA, France, Denmark, Australia and Canada, have accepted the use of enzymes from *Aspergillus niger* in a number of food applications.

Strains belonging to the *Aspergillus niger* GAM-lineage as well as the host (recipient) strain ISO-528 from DSM were declared suitable host strains for the construction of genetically modified organisms belonging to Group I safe micro-organisms by the Dutch authorities, as discussed in Section 2.1 (see Annex 2.1.1).

The *Aspergillus niger* GAM-53 strain, which was used as the parental strain of the host organism, has already been used as host for the selection of genetically modified production strains, by the process of random integration, for the production of the enzymes phytase and xylanase. DSM uses these strains on industrial scale since 1991 and 1996, respectively.

The recombinant acid lactase strain TOL-54 (DS 63558) has been classified by both the Dutch and French competent authorities as a Group I safe micro-organism, as discussed in Section 2.4 (See Annexes 2.4.2 and 2.4.3). Consequently, the strain was approved for large scale production of acid lactase in the DSM factory in Seclin, France.

Based on the genetic modification performed (see Section 2.2), there are no reasons to assume that the recombinant production strain should be less safe than the original GAM-53 (DS 3045) strain. In fact, it has been shown that the DSM GAM/ISO lineage of *A. niger* strains are safe hosts for the over-expression of enzymes to the extent that for new enzymes the safety is already covered by the safety studies performed on other enzyme production strains derived from this lineage and consequently new safety studies are superfluous (van Dijck et al., 2003, see Annex 2.1.1).

At the end of the fermentation, the recombinant production organism is effectively killed off (see Section 4.4).

Specific tests have been performed to confirm that the recombinant acid lactase production strain is not able to produce any toxins under the routine conditions of industrial submerged fermentations. The results of these tests showed that the production strain does not produce any known toxins under these conditions.

7.2 Safety of the Acid Lactase Enzyme

As noted above, enzymes produced by *Aspergillus niger* have already been used for food production for several decades. In the USA, FDA has previously affirmed as GRAS several enzyme preparations from *Aspergillus niger* and subsequently received GRAS notifications for additional enzyme preparations, including several produced from genetically modified *Aspergillus niger* strains, such as carbohydrases, proteases, pectinases, glucose oxidase and catalase (GRN 000089), lactase (GRN 000132), and lipase (GRN 000111 and GRN 000158). A publication authored by FDA professionals included a summary of the safety of microorganisms, including *Aspergillus niger*, used as a host for enzyme-encoding genes (Olempska-Beer, Z.S. et al., 2006). In addition, a phospholipase A₂ (GRN 000183), an asparaginase (GRN 000214), a

lipase (GRN 000296), a carboxypeptidase (GRN 000345) and a peroxidase (GRN 000402) preparation from genetically modified *Aspergillus niger* strains, which were derived from the same strain-lineage as the *Aspergillus niger* strain described in this dossier, have been notified as GRAS. FDA had no objections to each of these notifications.

The Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO has evaluated several enzymes from *Aspergillus niger* for their safety (Joint FAO/WHO Expert Committee on Food Additives, 1975a, Joint FAO/WHO Expert Committee on Food Additives, 1975b, Joint FAO/WHO Expert Committee on Food Additives, 1988). All these enzymes have received an ADI “not specified” by JECFA (see also Section 7.1).

Since it is generally known that commercial enzyme preparations of *Aspergillus niger* are not toxic and since acid lactase is a natural constituent of many organisms, including microorganisms, used for food (see Sections 6.4.2 and 7.2.2), it is not expected that acid lactase will have any toxic properties.

The enzyme preparation acid lactase produced by *Aspergillus niger* strain TOL-54, over-expressing the acid lactase gene from *Aspergillus oryzae*, was evaluated according the Pariza & Johnson Decision Tree. The decision tree is based on the safety evaluation methodology published by Pariza and Foster in a 1983 article, which was extended by the IFBC into the decision tree format and published in 1991. In 2001, Pariza and Johnson published an update. DSM’s decision tree analysis, based on the most recent update of the decision tree, is described in Annex 7.2. Based on the Pariza and Johnson decision tree analysis, DSM concludes that the acid lactase preparation is safe.

To confirm the above assumption that the acid lactase preparation does not have any toxic properties and to further establish the toxicological safety of the use of acid lactase from *Aspergillus niger* in food, the following studies were performed:

- Sub-chronic (90 day) oral toxicity study
- Ames test
- Chromosomal aberration test, *in vitro*
- Mammalian Erythrocyte Micronucleus Test, *in vivo*

No adverse effects, mutagenic or clastogenic activity were discovered in the studies, which are described in further detail in Section 7.4.1.

7.2.1 Allergenicity

Enzymes are proteins with highly specialized catalytic functions. They are produced by all living organisms and are responsible for many essential biochemical reactions in microorganisms, plants, animals, and human beings. Enzymes are essential for all metabolic processes and they have the unique ability to facilitate biochemical reactions without undergoing change themselves. As such, enzymes are natural protein molecules that act as very efficient catalysts of biochemical reactions.

As a protein, enzymes have the potential to cause allergic responses. Although virtually all allergens are proteins, it is noteworthy that only a small percentage of all dietary proteins are food allergens.

The unique role of enzymes in food processing is as a catalyst. Due to the specific nature of enzymes, only small amounts are required to make desired modifications to the property of a food. The use levels are based on the activity of the enzyme, not the amount of the enzyme product.

Enzymes have a long history of safe use in food. Since new enzymes are generally based on existing enzymes, it is very unlikely that a new enzyme would be a food allergen. Moreover, exposure of the enzyme associated with ingestion is typically very low and residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system (Grimble, G.K., 1994). To our knowledge no reports exist on sensitization to enzyme products in the final commercial food after ingestion.

The absence of food allergenicity has been confirmed by an extensive literature search and survey of producers' files, in which no cases have been found of people who have been sensitized or persons who experienced an allergic reaction following the ingestion of food prepared with various enzymes (see Annex 7.2.1). Even among people who ingest high daily doses of enzymes as digestive aids for many consecutive years, there are no reports of gastrointestinal allergy to enzymes. Recently, it was concluded that ingestion of food enzymes in general is not considered to be a concern with regards to food allergy (Bindeslev-Jensen, C. *et al.*, 2006).

For the purpose of this dossier the amino acid sequence of acid lactase was compared with the amino acid sequences of known (food) allergens stored in the database Allermatch™ (Fiers, M.W. *et al.*, 2004). The Allermatch database allows search in SwissProt database (last updated December 4th 2007) and WHO-IUIS database (last updated December 21st 2007). The allergenicity screening of the amino acid sequence of acid lactase has been performed in January 2009.

The results indicate that the sequence of acid lactase does not show 35% or more overlap with known allergens using a window of 80 amino acids and no matches of 8 amino acids or more are observed. Based on these results it is concluded that the acid lactase protein has no relevant match with known (food) allergens and is not likely to produce an allergenic or sensitization response upon oral consumption.

In addition, acid lactase has been used worldwide for a number of years without any complaints from the end consumer.

With regard to allergenicity of the fermentation media, DSM has concluded that the data that it has and the public data and information allow it to conclude that there is no published or unpublished data that suggest there is an allergen causing protein from the fermentation media in the finished enzyme product. To reach that conclusion, DSM relies on:

1. The Enzyme Technical Association in 2004 conducted a survey of its members, and collected information on the possible presence of protein from the fermentation media in the final enzyme product. ETA provided the supporting data and information to FDA in a letter in 2005, and sent an accompanying public statement which is posted on ETA's website. The statement concludes that no allergens protein from the fermentation medium has been found in the finished enzyme, and states that regulatory bodies in both the EU and Japan have concluded that enzyme preparations do not pose an allergen risk that would require allergen labeling on the final product. Further, ETA points out that the typical manufacturing process of enzyme preparations includes a step to separate the biomass and fermentation media from the enzyme. This step ensures the enzyme product purity and stability, and would likely remove most proteins present in the fermentation media. A copy of the public statement from the ETA website is attached in Annex 7.2.1.A.

2. In addition, the Food Allergy Research and Resource Program (FARRP) issued a paper in August of 2013 which concluded that because of the nature of enzymes as catalysts, they are used in very small amounts and the fermentation media is consumed during the enzymatic process. It is clear that any de minimis amount of fermentation media protein that survived the fermentation process will not cause a significant public health risk to the consumer. FARRP also underscores the fact that the proteins would likely be removed during the filtration of the enzyme product, as discussed by ETA. Further, FARRP indicates that there is no reliable assay that could be used to detect the presence of most allergen proteins in the final enzyme products, as the proteins would likely be degraded fragments that would not reach levels of quantitation available with current commercial ELISA assays. The full August 2013 statement, provided in Annex 7.2.1.B, clearly concludes that any protein allergen present in the final enzyme product would not be present at a level that requires labeling or present at a level that raises a public health concern.

3. In addition, DSM has data from a study where wheat derived carbohydrates were used during fermentation, and an analysis after the fermentation shows the absence of gluten despite a detection limit of 10 parts per million (ppm). Finally, soy flour was used as a fermentation media, and post-fermentation analysis of the enzyme product revealed that no soy residue was present, with a level of detection of 0.5 ppm.

Finally, it is our understanding that a search of the scientific literature will not result in a reported allergic reaction from an enzyme caused by the fermentation medium. The ETA has conducted similar literature searches in the past, with no findings of allergic reactions due to fermentation media. The fermentation media as noted above is consumed in the process, and is removed with subsequent purification and filtration steps used in the enzyme production process. There is no evidence to support that a level of protein from the fermentation media exists in the final enzyme product which would cause an allergic reaction.

It is therefore concluded that the acid lactase protein is not likely to produce any allergenic or sensitization reactions by oral consumption.

7.2.2 Leading Enzyme Publications on the Safety of Acid Lactase Enzymes or Enzymes that are Closely Related

The safety of the production organism is the point of focus as to the safety of the enzyme which will be used in food processing. In this case, the production organism *Aspergillus niger*, has been demonstrated to be nonpathogenic and any food ingredient (enzyme) from that organism will exhibit the same safety properties if manufactured under current Good Manufacturing Practices (“cGMPs”). Pariza and Foster (1983) noted that a nonpathogenic organism was very unlikely to produce a disease under ordinary circumstances. In their publication, the authors include a list of the organisms being used in the industry of which *Aspergillus niger* is one.

The FDA has also accepted GRAS Notifications from the Enzyme Technical Association and DSM Food Specialties stating that carbohydrase, pectinase, protease, glucose oxidase and catalase (GRN 000089), lipase (GRN 000111; GRN 000158; GRN 000296), lactase (GRN 000132), phospholipase A2 (GRN 000183), asparaginase (GRN 000214), carboxypeptidase (GRN 000345) and peroxidase (GRN 000402) enzyme preparations from *Aspergillus niger* are generally recognized as safe. *Aspergillus niger* is listed as a production organism for enzymes (Pariza, M.W. and Johnson, E.A., 2001) and has a long history of safe use (see Annex 7.1.1).

As is clear from the information provided in this notification, there have been genetic modifications to the *Aspergillus niger* used by DSM, but these genetic modifications are thoroughly well characterized and specific in that the DNA encoded does not express any harmful or toxic substance. The safety studies described in Section 7.4 of this dossier support the fact that the genetic modification did not result in any toxic effects.

The evaluation of the safety of the genetic modification should be examined based on the concepts outlined in the Pariza and Foster (1983) paper. Their basic concepts were further developed by the IFBC in 1990, the EU Scientific Committee for Food in 1991, the OECD in 1991, ILSI Europe Novel Food Task Force in 1996 and FAO/WHO in 1996. Basically, the components of these evaluations start with an identified host strain, descriptions of the plasmid used and the source and fraction of the material introduced, and an outline of the genetic construction of the production strain. This information is found in Section 2.

Acid lactases are ubiquitous in nature and can be found in fungi, plants and animals, including humans (see Section 6.4.2). They have been recognized as an individual category by the International Union of Biochemistry and Molecular Biology (IUBMB) since 1961.

Acid lactases are glycosidases that hydrolyze O- and S-glycosyl compounds. Lactases have been affirmed as GRAS by FDA in 2003 (GRN 000132 and GRN 000088) and they have been included in the safety evaluation by Pariza and Johnson (2001).

As Distler described in 1978, Hemavathi in 2008, Konno in 1988 and Kaneko in 2003, cows, barley, carrots and rice all contain acid lactases and as such, naturally-occurring acid lactases are consumed as they will be in the applications described here.

7.2.3 Substantial Equivalence

Several expert groups have discussed the concept of substantial equivalence relative to food safety assessment. Essentially, all these groups conclude that if a food ingredient is substantially equivalent to an existing food ingredient known to be safe, then no further safety considerations other than those for the existing ingredient are necessary.

In addition, FDA appears to have accepted this concept in the determination that several enzyme preparations are safe for use in food. In particular, FDA has considered differences in glycosylation between enzyme proteins. FDA has also stated that enzyme proteins demonstrated to be substantially equivalent to enzymes known to be safely consumed but having differences in specific properties due to chemical modifications, or site-direct mutagenesis, would not raise safety concerns.

There are no agreed-upon criteria by which substantial equivalence is determined. Considering enzymes produced by micro-organisms the enzyme activity and intended use, the production organism and the process conditions should be taken into account.

As indicated in Section 3.3, close similarities exist between the acid lactase that is the subject of the present GRAS notification and other beta-galactosidases that have been safely marketed for years, including those that are the subject of GRAS notifications.

Lactases (IUB 3.2.1.23) are part of the glycosidases (IUB 3.2.1). Two other lactases have been notified as GRAS by the FDA: Lactase enzyme preparation from *Kluyveromyces marxianus* (GRN 000088) and Lactase enzyme preparation from *Aspergillus niger* (GRN 000132). To this notification, FDA responded with a letter stating they had no questions.

In addition to the safety of the acid lactase enzyme itself, the current production strain is derived from a safe strain lineage of *Aspergillus niger*. *Aspergillus niger* is a common food constituent of products like rice, seeds, nuts, olives and dried fruits. In addition, *Aspergillus niger* has been used for several decades for the production of organic acids and enzymes to be used in the food industry. The FDA recently summarized the safety of microorganisms, including *Aspergillus niger*, used as a host for enzyme-encoding genes (Olempska-Beer, Z.S. *et al.*, 2006).

Other food substances from *Aspergillus niger* were previously affirmed as GRAS. See 21 C.F.R. § 184.1033 (Citric acid); 184.1685 (Rennet and chymosin). Also, the FDA subsequently received GRAS notifications for additional enzyme preparations from *Aspergillus niger*, including several produced from genetically modified *Aspergillus niger* strains, such as carbohydrases, proteases, pectinases, glucose oxidase and catalase (GRN 000089), lactase (GRN 000132) and lipase (GRN 000111 and GRN 000158). FDA has no questions with these GRAS notifications.

The Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO has evaluated several enzymes from *Aspergillus niger* for their safety (Joint FAO/WHO Expert Committee on Food Additives, 1975a, Joint FAO/WHO Expert Committee on Food Additives, 1975b, Joint

FAO/WHO Expert Committee on Food Additives, 1988). All these enzymes have received an ADI “not specified” by JECFA (see also Section 7.1).

Finally, the safety of the *Aspergillus niger* strain lineage that is used to produce DSM’s acid lactase has been extensively described by (Van Dijck, P.W.M. *et al.*, 2003). The publication describes the standardized method of producing food enzymes. The production process, the production strain, the construction of the production strain, and the raw materials used in the fermentation and downstream processing are kept the same. Only the gene encoding the enzymatic activity is changed. Extensive toxicological studies of a number of strains built and processed according to this method demonstrate the safety of the enzymes produced this way. The acid lactase-producing strain described in this dossier was built and produced according to this method.

Moreover, a phospholipase A₂ (GRN 000183), an asparaginase (GRN 000214), a lipase (GRN 000296), a carboxypeptidase (GRN 000345) and a peroxidase (GRN 000402) preparation from genetically modified *Aspergillus niger* strains, derived from the same strain-lineage as the *Aspergillus niger* strain described in this dossier and processed according to the mentioned concept, were the subject of GRAS notifications to which FDA has no questions.

Thus, the *Aspergillus niger* production strain used to produce acid lactase is as safe as the production strains that have produced other GRAS enzymes subject to GRAS notifications. Accordingly, it can be concluded that the resulting enzyme product from the production strain is as safe as other enzymes produced by strains from the same safe strain lineage and processed the same way.

Also the use of the acid lactase described here is substantially equivalent to already described enzymes like lactase or beta-galactosidase. This can be seen in peer-reviewed articles (Woychik, J. and Holsinger, V., 1977, Mlichová, Z. and Rosenberg, M., 2006).

Since the production strain and production process are as safe as those used to produce other GRAS enzymes, and the acid lactase itself is substantively similar to other GRAS galactosidase enzymes in terms of activity and intended use, it can be concluded that the acid lactase is GRAS.

7.3 Safety of the Manufacturing Process

Acid lactase meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex. As described in Section 5.2, the acid lactase preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for general use in foods, under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for the production of microbial enzymes.

7.4 Safety Studies

This section describes the studies performed to evaluate the safety of using DSM’s acid lactase preparation.

7.4.1 Safety Studies in Summary

All safety studies were performed in compliance with the principles of Good Laboratory Practice (GLP) according to the FDA/OECD.

The oral toxicity studies were performed on the diafiltrate concentrate (ccDF) batch TLD/C717001/E, referred to as 'tox-batch', by oral gavage. The tox-batch was produced according to the procedure used for commercial production and represents the commercial product. The non-formulated tox-batch had an activity of 30400 ALU/g and a TOS content of 15.5%. The Certificate of Analysis is included in the sub chronic oral toxicology report.

90-day oral toxicity

A sub-chronic oral toxicity study with the tox-batch was conducted at Advinus, India, in accordance with the following guidelines:

- OECD Guideline for the Testing of Chemicals 408. Repeated Dose 90-day Oral Toxicity Study in Rodents, adopted 21st September 1998.
- B.26. Sub chronic oral toxicity test. Repeated dose 90-day oral toxicity study in rodents. Annex 5D to Commission Directive 2001/59/EC, Official Journal of the European Communities L225, 21.8.2001.

The study consisted of four groups of 10 male and 10 female Wistar rats, one control group and three test groups (low, mid and high dose). For a period of 90 days, the three test groups received 645 mg, (low-dose), 1935 mg (mid-dose) and 6452 mg (high-dose) of enzyme preparation of *Aspergillus niger* containing acid lactase activity, respectively, by oral gavage. The animals in the control group received vehicle (water) only, all groups were dosed at a volume of 10 ml/kg body weight/day. The low, mid, and high dose correspond with respectively 100, 300 and 1000 mg TOS (Total Organic Solids)/kg body weight/day.

General clinical observations, neurobehavioral testing, ophthalmoscopic examination, body weight, food consumption, hematology, clinical chemistry, organ weights of principal organs, macroscopic examination, histopathology of organs (control and high-dose group only) and histopathology of all lesions were studied.

Results

The administration of the tox-batch at dietary levels up to 6452 mg of enzyme preparation did not lead to any toxicologically relevant findings. The NOAEL is therefore 6452 mg of enzyme preparation, the highest dose level tested. This corresponds to 1000 mg TOS/kg body weight/day or 196130 U/kg body weight/day.

Mutagenicity tests

AMES test

A bacterial reverse mutation test was performed with the tox-batch at Notox, the Netherlands, in order to assess its mutagenic activity in four selected strains of *Salmonella typhimurium*, TA 1535, TA 1537, TA 98 and TA 100, as well as in the *Escherichia coli* mutant WP₂uvrA. All were tested in both the absence and presence of a metabolic activation system (S9-mix).

The study was conducted in accordance with the following guidelines:

- OECD guideline no. 471, Genetic toxicology: Bacterial Reverse Mutation Test, adopted 21st July 1997.
- European Community (EC). Commission Regulation (EC) No. 2008/440/EC: Part B: Methods for the Determination of Toxicity and other health effects; Guideline B.13/14: Mutagenicity: Reverse Mutation Test using Bacteria. Official Journal of the European Union No L142, 31. May 2008

Enzyme preparation of *Aspergillus niger* containing acid lactase activity was tested in the *Salmonella typhimurium* reverse mutation assay with four histidine-requiring strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and in the *Escherichia coli* reverse mutation assay with a tryptophan-requiring strain of *Escherichia coli* (WP₂uvrA). The test was performed in two independent experiments in the presence and absence of S9-mix (rat liver S9-mix induced by a combination of phenobarbital and β -naphthoflavone) with five concentrations of the tox-batch, ranging from 100 to 5000 mg TOS/plate.

Enzyme preparation of *Aspergillus niger* containing acid lactase activity did not induce a significant dose-related increase in the number of revertant (His⁺) colonies in each of the four tester strains (TA1535, TA1537, TA98 and TA100) and in the number of revertant (Trp⁺) colonies in tester strain WP₂uvrA both in the absence and presence of S9-metabolic activation. These results were confirmed in an independently repeated experiment.

In this study, the negative and strain-specific positive control values were within the laboratory historical control data ranges indicating that the test conditions were adequate and that the metabolic activation system functioned properly.

Based on the results of this study it is concluded that Enzyme preparation of *Aspergillus niger* containing acid lactase activity is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

Chromosomal aberration test

A chromosomal aberration test *in vitro* was performed with the tox-batch at Notox, the Netherlands, in order to assess its ability to induce structural chromosomal aberrations in cultured human lymphocytes in the absence and presence of a metabolic activation system (S9-mix).

The study was conducted in accordance with the following guidelines:

- OECD guideline 473, Genetic toxicology: *In vitro* Mammalian Chromosome Aberration Test, adopted 21st July 1997.
- European Community (EC). Commission Regulation (EC) No. 2008/440/EC: Part B: Methods for the Determination of Toxicity and other health effects; Guideline B.10: “Mutagenicity – *in vitro* Mammalian Chromosome Aberration test”. Official Journal of the European Union No L142, 31. May 2008.

This effect of an enzyme preparation of *Aspergillus niger* containing acid lactase activity on the number of chromosome aberrations in cultured peripheral human lymphocytes was assessed both in the presence and absence of a metabolic activation system (phenobarbital and β -naphthoflavone induced rat liver S9-mix). The possible clastogenicity of the enzyme product was tested in two independent experiments.

In the first cytogenetic assay, an enzyme preparation of *Aspergillus niger* containing acid lactase activity was tested up to 5000 μ g TOS/ml for a 3 h exposure time with a 24 h fixation time in the absence and presence of 1.8% (v/v) S9-fraction. This is the highest concentration that should be tested according to the guidelines.

In the second cytogenetic assay, an enzyme preparation of *Aspergillus niger* containing acid lactase activity was tested up to 3000 μ g TOS/ml for a 24 h continuous exposure time with a 24 h fixation time and up to 5000 μ g TOS/ml for a 48 h continuous exposure time with a 48 h fixation time in the absence of S9-mix. Appropriate toxicity was reached at the highest dose level tested of the 24 h exposure time. In the presence of S9-mix enzyme preparation of *Aspergillus niger* containing acid lactase activity was tested up to 5000 μ g TOS/ml for a 3 h exposure time with a 48 h fixation time.

The number of cells with chromosome aberrations found in the solvent control cultures was within the laboratory historical control data range. Positive control chemicals, mitomycin C and cyclophosphamide, both produced a statistically significant increase in the incidence of cells with chromosome aberrations, indicating that the test conditions were adequate and that the metabolic activation system (S9-mix) functioned properly.

Enzyme preparation of *Aspergillus niger* containing acid lactase activity did not induce a statistically significant or biologically relevant increase in the number of cells with chromosome aberrations in the absence and presence of S9-mix, in either of the two independently repeated experiments.

It was noted that the enzyme preparation of *Aspergillus niger* containing acid lactase activity increased the number of polyploid cells in the first cytogenetic assay both in the absence and presence of S9-mix in a dose dependent manner. This may indicate that the enzyme preparation of *Aspergillus niger* containing acid lactase activity has the potential to inhibit mitotic processes and to induce numerical chromosome aberrations.

No effects of the enzyme preparation of *Aspergillus niger* containing acid lactase activity on the number of cells with endoreduplicated chromosomes were observed both in the absence and presence of S9-mix.

Finally, it is concluded that this test is valid and that the enzyme preparation of *Aspergillus niger* containing acid lactase activity is not clastogenic in human lymphocytes under the experimental conditions described. The enzyme preparation of *Aspergillus niger* containing acid lactase activity may have the potential to inhibit mitotic processes and to induce numerical chromosome aberrations.

Mouse micronucleus test

To further clarify the increased number of polyploidy cells detected in the *in vitro* chromosome aberration test and to determine if the enzyme preparation of *Aspergillus niger* containing acid lactase activity may have the potential to inhibit mitotic processes and to induce numerical chromosome aberrations, an *in vivo* micronucleus assay was performed by WIL Research, The Netherlands. The *in vivo* study has been chosen because it is judged to have more weight in the evaluation of a product than *in vitro* studies.

The tox-batch was examined for its potential to induce mutagenic effects in mice following acute oral administration using an *in vivo* cytogenetic system. The oral route was chosen for this particular study as the likely route of human exposure to the test substance.

Mice were treated with a single oral administration of the tox-batch at dose levels of 500, 1000 and 2000 mg/kg body weight. A preliminary toxicity test had previously shown that a dose of 2000 mg/kg, the limit dose for the micronucleus test, was tolerated; this level was therefore selected as an appropriate maximum. The preliminary toxicity test also showed no difference in toxicity between the sexes and in line with guidelines, only male animals were used in the micronucleus test.

The tox-batch and negative control were administered with two consecutive doses via oral gavage. The negative control group received the vehicle, purified water. A positive control group was dosed once via oral gavage, with cyclophosphamide (CP) at 40 mg/kg body weight.

Bone marrow of the groups treated with the enzyme preparation from *Aspergillus niger* containing acid lactase activity was sampled 24 or 48 (highest dose only) hours after dosing. Bone marrow of the negative and positive control groups was harvested 24 and 48 hours after dosing, respectively.

No increase in the mean frequency of micronucleated polychromatic erythrocytes was observed in the bone marrow of animals treated with Enzyme preparation from *Aspergillus niger* containing acid lactase activity. The incidence of micronucleated polychromatic erythrocytes in the bone marrow of all negative control animals were within the historical vehicle control data range. Cyclophosphamide, the positive control substance, induced a statistically significant increase in the number of micronucleated polychromatic erythrocytes. Hence, both criteria for an acceptable assay were met.

The groups that were treated with the enzyme preparation from *Aspergillus niger* containing acid lactase activity showed no decrease in the ratio of polychromatic to normochromatic erythrocytes compared to the concurrent vehicle control group, indicating a lack of toxic effects of this test substance on erythropoiesis. The group that was treated with cyclophosphamide showed an expected decrease in the ratio of polychromatic to normochromatic erythrocytes compared to the vehicle control, demonstrating toxic effects on erythropoiesis.

It was concluded that the enzyme preparation from *Aspergillus niger* containing acid lactase activity is not clastogenic or aneugenic in the bone marrow micronucleus test when sampled at 24 and 48 hours post dosing of male mice up to a dose of 2000 mg TOS/kg (the maximum recommended dose in accordance with current regulatory guidelines) under the experimental conditions described in this report.

Therefore, based on the analysis of the results of both the *in vitro* chromosome aberration test and the *in vivo* mouse micronucleus test, the enzyme preparation from *Aspergillus niger* containing acid lactase activity does not appear to be clastogenic.

7.5 Estimates of Human Consumption and Safety Margin

The maximum amount of enzyme present in the final food is 28,000 ALU/l milk (see Section 6.3).

The Estimated Daily Intake (EDI) was calculated based on maximum dose levels and consumption data of milk and milk products in the United States (Wilkinson Enns, C. *et al.*, 1997). Moreover, FDA estimates a daily consumption of 3.8 g cheese-whey products/person in the USA (GRAS notification GRN 000037).

Final food	Maximum amount of enzyme in final food (per liter)		90 th percentile intake level (gram/person/day) ¹	Estimated daily intake of (denatured) enzyme per kg bw ²	
	ALU	TOS (mg)		ALU	TOS (mg)
Milk and Milk products ³	28,000	157	560	261	1.4653
Whey based products	22,400	125	7.6	2.8	0.016
Total				264	1.4813

1. 90th percentile is 2 times the intake level (CFR 201.10, 2006).

2. Calculated for a person of 60 kg.

3. Derived from the category 'milk and milk products'. The amount of enzyme is based on the amount of lactose, therefore, the application dose in milk is used to calculate the intake of (denatured) enzyme.

The Margin of safety can be calculated by dividing the NOAEL by the EDI, i.e. 1000 mg TOS/kg bw /day / 1.4813 mg TOS/kg bw/day = 675.

The height of the Margin of Safety confirms the safety of the product.

7.6 Results and Conclusion

Results of the toxicity, mutagenicity and clastogenicity tests described in Section 7.4.1 demonstrate the safety of DSM's acid lactase preparation, which showed no toxicity, mutagenicity or clastogenicity across a variety of test conditions. The data resulting from these studies are consistent with the long history of safe use for *Aspergillus niger* in food processing, the natural occurrence of lactase in foods, and data presented in relevant literature. Based upon these factors, as well as upon the limited and well-characterized genetic modifications allowing for safe production of the acid lactase preparation, it is DSM's conclusion that acid lactase preparation from *Aspergillus niger* is GRAS for the intended conditions of use.

8 LIST OF ANNEXES

- 2.1.1 Letter from Director-General Milieu to DSM's Strain Director, dated 06-07-2005
- 2.1.2 Letter from CBS to Gist-Brocades, dated 29.1.1994 regarding Identification Service
- 2.1.3 Dijck, P.W.M. van, Selten, G.C.M., Hempenius, R.A., On the safety of a new generation of DSM *Aspergillus niger* enzyme production strains, Regulat. Toxicol. Pharmacol. 38:27-35 (2003)
- 2.1.4 Letter from Director-General Milieu to DSM's Strain Director, dated 06-07-2005
- 2.4.1 Surviving studies of *Aspergillus niger* strains in soil, surface water, and waste water
- 2.4.2 Letter from Director-General Milieu to DSM Strain Director, dated 1 December 2009
- 2.4.3 Letter from the Comite Scientifique du Haut Conseil des Biotechnologies dated 2 March 2010
- 3.2.1 Amino acid sequence of acid lactase from *Aspergillus niger*
- 4.1.1 Flow diagram of manufacturing process
- 7.1.1 Schuster, E., Dunn-Coleman, N., Frisvad, J.C., Dijck, P.W.M van, *On the Safety of Aspergillus niger – a Review*, Appl. Microbiol. Biotechnol. 59:426-435 (2002)
- 7.1.2 JECFA safety evaluation of *Aspergillus niger* as a source of enzymes to be used in food, 1987
- 7.1.3 Expert reports of Prof. J.W. Bennett and Dr. M.O. Moss on the probability of mycotoxins being present in industrial enzyme preparations obtained from fungi (September 1988)
- 7.1.4 JECFA reconsideration of safety of *Aspergillus niger* as a source for enzymes to be used in food, 1990
- 7.2 Safety evaluation using the Pariza & Johnson decision tree of acid lactase from *Aspergillus niger* TOL-54
- 7.2.1 Literature search on allergenicity by ingestion of food prepared with enzymes
- 7.2.1.A Statement from the Enzyme Technical Association
- 7.2.1.B Food Allergy Research and Resource Program paper

9 LIST OF REFERENCES

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Annex 2.1.1

VROM (Ministry of Housing
Spatial Planning and the Environment)

Directorate-General for Environmental Protection
Direction Substances, Safety and Radiation
Department Radiation, Nuclear and Biosafety

DSM Anti-Infectives B.V.
Attn.: Dr. P.W.M. van Dijck, DSM Strain Director
Internal post-code 001-0490
P.O. Box 1
2600 MA DELFT

RIVM/SEC/Bureau GGO
Anthonie van Leeuwenhoeklaan 9

P.O.Box 1
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Tel: 030-2744197
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URL : www.rivm.nl/sec/bggo_nl.html

Decree new application : EVO 05-010

Date	Reference	Attachments
06-07-2005	EVO 05-010.bes	
Your letter 06-06-2005	Your reference PWMvD/REG#46808	Copies to: Commission Genetic Modification (COGEM)

Dear Board of Management,

Herewith I send you a permit based on the Decree Genetically Modified Organisms Act Environmentally dangerous substances (Official Journal 1993, 435), with the number EVO 05-010.

Interested parties may oppose to this decision, within six weeks after it has been send, based on the General Administrative Law, by submitting a motivated petition to the State Secretary of Housing, Spatial Planning and the Environment. The petition has to be addressed to: The State Secretary of VROM attn. RIVM/SEC/Bureau GGO, P.O. Box 1, 3720BA Bilthoven. The petition has to bear a date and a name and address. It must be shown clearly why the decision is petitioned and when possible a copy of the decision should be included.

Sincerely,

Ing. C.H. Roesink
Bureau GGO (Bureau for GMO Affairs)

VROM (Ministry of Housing
Spatial Planning and the Environment)

Directorate-General for Environmental Protection
Direction Substances, Safety and Radiation
Department Radiation, Nuclear and Biosafety

Rijnstraat 8
P.O. Box 30945
2500GX The Hague
Internal postcode 645

www.vrom.nl

Decree
DGM/SAS EVO 05-010/00

Having read the application of DSM Anti-Infectives B.V., in Delft, of 06-06-2005, reference:
PWMvD/REG#46808,

The State Secretary of Housing, Spatial Planning and the Environment,

Considering, the provisions in the Decree genetically modified organisms Law on
Environmentally dangerous substances (Official Journal 1993, 435), article 2 of this Decree
and the provisions of the Regulation Genetically Modified Organisms (Official Journal 1998,
108), article 3 of this Regulation,

Decides:

The strain *Aspergillus niger* ISO 528 is approved for the construction of genetically modified
organisms belonging to group I with which activities of both category A and B may be carried
out.

The Hague, 05-07-2005

The State Secretary of Housing, Spatial Planning and the Environment,
On his behalf, the Director-general Environmental Control
b.o. The Head of the department Radiation, Nuclear- and Biosafety

Mr. A. van Limborgh



Directoraat-Generaal Milieu
Directie Stoffen, Afvalstoffen, Straling
Afdeling Straling, Nucleaire en Bioveiligheid

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DSM Anti-Infectives B.V.
T.a.v. dr. P.W.M. van Dijk, DSM Strain Director
postpunt 001-0490
Postbus 1
2600 AM Delft

Beschikking nieuwe aanvraag: EVO 05-010

Datum	Kenmerk	Bijlagen
06-07-05	EVO 05-010.bes	
Uw brief	Uw kenmerk	Afschrift aan
06-06-2005	PWMvD/REG#46808	Commissie Genetische Modificatie

Geachte Directie,

Hierbij zend ik u een vergunning op grond van het Besluit genetisch gemodificeerde organismen Wet Milieugevaarlijke Stoffen (Stb. 1993, 435), met nummer EVO 05-010.

Tegen deze vergunning kunnen belanghebbenden binnen zes weken na verzending van de beschikking op grond van de Algemene wet bestuursrecht een gemotiveerd bezwaarschrift indienen bij de Staatssecretaris van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer. Het bezwaarschrift moet gezonden worden aan: De Staatssecretaris van VROM, t.a.v RIVM/SEC/Bureau GGO, postbus 1, 3720 BA Bilthoven. Een bezwaarschrift moet van een datum en een naam en adres voorzien zijn. Er moet duidelijk worden aangegeven waarom tegen de vergunning bezwaar wordt aangetekend en zo mogelijk wordt een kopie van de vergunning meegezonden.

Hooachtend,

(b) (6)

ing. C.H. Roesink
Bureau GGO

Annex 2.1.2

Ministry of Housing
Spatial Planning and the Environment

Post-box 30945
2500 GX The Hague
Internal post-code 655
Tel: 070-3394866
Fax: 070-3391297

Directorate-General for Environmental Protection
Direction Substances, Safety and Radiation

To: Gist-broccades
Dr. A. de Leeuw
Post-box 1
2600 MA DELFT

Your reference
AdL/PP/94-111

Your letter
11/11/94

Reference
GGO94-g19

Date
7 December 1994

Subject

Application group I organisms
GGO 94-g19

Dear mister De Leeuw,

In connection with your application with the above date and reference I am reporting you the following:

1. The *Aspergillus niger* strains GAM-4, GAM-11, GAM-15, GAM-20, GAM-23, and GAM-53 are non-pathogenic and do not contain biological agents which are potentially harmful.
2. The *Aspergillus niger* strains GAM-4, GAM-11, GAM-15, GAM-20, GAM-23, and GAM-53 have a long history of safe use under conditions not exceeding the GILSP level of physical containment.

On the basis of these data and considerations I come to the conclusion that the *Aspergillus niger* strains GAM-4, GAM-11, GAM-15, GAM-20, GAM-23, and GAM-53 are suitable hosts for the construction of genetically modified organisms belonging which belong to group I and with which activities of both category A (lab-scale) and B (production-scale) may be carried out.

The Minister of Housing, Spatial Planning and the Environment,
On his behalf: the deputy Director-general Environmental Protection,
For him, the Director Substances, Safety, Radiation,

Dr. C.M. Plug

cc: - HIMH
- COGEM
- Ir. G.A. Harrewijn, BVF

enclosures



Ministerie van Volkshuisvesting,
Ruimtelijke Ordening en Milieubeheer

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2500 GX Den Haag
Interne Postcode 655
Tel : 070-3394866
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Directoraat-Generaal Milieubeheer
Directie Stoffen, Veiligheid, Straling
Stoffen

Gist-Brocades
dr. A. de Leeuw
Postbus 1
2600 MA DELFT

Uw kenmerk	Uw brief	Kenmerk	Datum
AdL/PP/94-111	11/11/94	GGO94-g19	7 december 1994
Onderwerp			
Aanvraag groep I organismen GGO 94-g19			

Geachte heer De Leeuw,

Naar aanleiding van uw aanvraag met bovenvermelde datum en kenmerk bericht ik u het volgende.

- 1 De *Aspergillus niger* stammen GAM-4, GAM-11, GAM-15, GAM20, GAM-23 en GAM-53 zijn niet-pathogeen en bevatten geen biologische agentia die potentieel schadelijk zijn.
- 2 De *Aspergillus niger* stammen GAM-4, GAM-11, GAM-15, GAM20, GAM-23 en GAM-53 hebben een lange historie van veilig gebruik onder omstandigheden die het GILSP niveau niet te boven gaan.

Op grond van deze gegevens en overwegingen kom ik tot de conclusie de *Aspergillus niger* stammen GAM-4, GAM-11, GAM-15, GAM20, GAM-23 en GAM-53 geschikte gastheren zijn voor het vervaardigen van genetisch gemodificeerde organismen die behoren tot groep I waarmee activiteiten van zowel categorie A als B mogen worden uitgevoerd.

De Minister van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer,
voor deze: de plv. directeur-generaal Milieubeheer.
o.l. de directeur Stoffen, Veiligheid, Straling.

(b) (6)

dr. C.M. Plug

c.c.: - HIMH
- VCOGEM
-ir. G.A. Harrewijn, BVF

Bijlagen

Annex 2.1.3

Pages 000064-000072 have been removed in accordance with copyright laws. The removed reference is:

Dijck, P.W.M. van, Selten, G.C.M., Hempenius, R.A., On the safety of a new generation of DSM *Aspergillus niger* enzyme production strains, Regulat. Toxicol. Pharmacol. 38:27-35 (2003)

Annex 2.1.4

VROM (Ministry of Housing
Spatial Planning and the Environment)

Directorate-General for Environmental Protection
Direction Substances, Safety and Radiation
Department Radiation, Nuclear and Biosafety

DSM Anti-Infectives B.V.
Attn.: Dr. P.W.M. van Dijck, DSM Strain Director
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URL : www.rivm.nl/sec/bggo_nl.html

Decree new application : EVO 05-011

Date	Reference	Attachments
06-07-2005	EVO 05-011.bes	
Your letter	Your reference	Copies to:
06-06-2005	PWMvD/REG#46808	Commission Genetic Modification (COGEM)

Dear Board of Management,

Herewith I send you a permit based on the Decree Genetically Modified Organisms Act Environmentally dangerous substances (Official Journal 1993, 435), with the number EVO 05-013.

Interested parties may oppose to this decision, within six weeks after it has been send, based on the General Administrative Law, by submitting a motivated petition to the State Secretary of Housing, Spatial Planning and the Environment. The petition has to be addressed to: The State Secretary of VROM attn. RIVM/SEC/Bureau GGO, P.O. Box 1, 3720BA Bilthoven. The petition has to bear a date and a name and address. It must be shown clearly why the decision is petitioned and when possible a copy of the decision should be included.

Sincerely,

Ing. C.H. Roesink
Bureau GGO (Bureau for GMO Affairs)

VROM (Ministry of Housing
Spatial Planning and the Environment)

Directorate-General for Environmental Protection
Direction Substances, Safety and Radiation
Department Radiation, Nuclear and Biosafety

Rijnstraat 8
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www.vrom.nl

Decree
DGM/SAS EVO 05-011/00

Having read the application of DSM Anti-Infectives B.V., in Delft, of 06-06-2005, reference:
PWMvD/REG#46808,

The State Secretary of Housing, Spatial Planning and the Environment,

Considering, the provisions in the Decree genetically modified organisms Law on
Environmentally dangerous substances (Official Journal 1993, 435), article 4.2 of this Decree
and the provisions of the Regulation Genetically Modified Organisms (Official Journal 1998,
108), article 3 of this Regulation,

Decides:

The genetically modified strain *Aspergillus niger* ISO-528 may be considered as a strain
obtained through self-cloning.

The Hague, 05-07-2005

The State Secretary of Housing, Spatial Planning and the Environment,
On his behalf, the Director-general Environmental Control
b.o. The Head of the department Radiation, Nuclear- and Biosafety

Mr. A. van Limborgh



Directoraat-Generaal Milieu
Directie Stoffen, Afvalstoffen, Straling
Afdeling Straling, Nucleaire en Bioveiligheid

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Beschikking nieuwe aanvraag: EVO 05-011

Datum 06-07-05	Kenmerk EVO 05-011.bes	Bijlagen
Uw brief 06-06-2005	Uw kenmerk PWMvD/REG#46808	Afschrift aan Commissie Genetische Modificatie

Geachte Directie,

Hierbij zend ik u een vergunning op grond van het Besluit genetisch gemodificeerde organismen Wet Milieugevaarlijke Stoffen (Stb. 1993, 435), met nummer EVO 05-011.

Tegen deze vergunning kunnen belanghebbenden binnen zes weken na verzending van de beschikking op grond van de Algemene wet bestuursrecht een gemotiveerd bezwaarschrift indienen bij de Staatssecretaris van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer. Het bezwaarschrift moet gezonden worden aan: De Staatssecretaris van VROM, t.a.v RIVM/SEC/Bureau GGO, postbus 1, 3720 BA Bilthoven. Een bezwaarschrift moet van een datum en een naam en adres voorzien zijn. Er moet duidelijk worden aangegeven waarom tegen de vergunning bezwaar wordt aangetekend en zo mogelijk wordt een kopie van de vergunning meegezonden.

Hooftachtend.

(b) (6)

ing. C.H. Roesink
Bureau GGO



beschikking

DGM/SAS EVO 05-011/00

Gelezen de aanvraag van DSM Anti-Infectives B.V., te Delft, van 06-06-2005, kenmerk: PWMvD/REG#46808,

De Staatssecretaris van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer,

Overwegende,
het bepaalde in het Besluit genetisch gemodificeerde organismen Wet milieugevaarlijke stoffen (Staatsblad 1993, 435), artikel 2 van dit besluit en het bepaalde in de Regeling genetisch gemodificeerde organismen (Staatscourant 1998, 108), artikel 3 van die regeling,

Besluit:

De genetisch gemodificeerde stam *Aspergillus niger* ISO-528 kan worden beschouwd als een stam verkregen door zelfklonering.

Den Haag, 05-07-2005

De Staatssecretaris van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer,
Voor deze,
de directeur-generaal Milieubeheer,
o.l.,

het hoofd van de afdeling Straling, Nucleaire en Bioveiligheid

(b) (6)



mr. A. van Limborgh

Annex 2.4.1

Surviving studies of *Aspergillus niger* strains in soil, surface water, and waste water.

Aspergillus niger was inoculated in three different environments, namely: soil, surface water and sewage (waste water of Gist-brocades production plant in Delft (The Netherlands)).

As positive control, that is to say to judge whether the environment contained sufficient substrates for the organisms to survive in the absence of competitive (micro)organisms, and that there were no toxic components present, additional inoculations were performed in sterile environments. Sampling was performed during 6 months.

Experimental results are shown in this annex (see figures 1,2 and 3, in which the data are presented for each environment at 8°C as well as 25°C.

In independent incubations the wild-type *Aspergillus niger* (NRRL 3122) and an industrial strain (GAM-53) were compared in sterile and non-sterile environments.

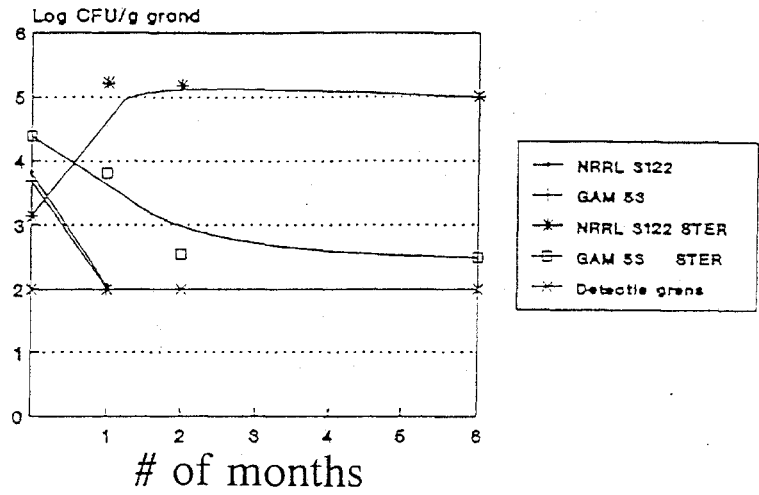
In all sterile environments at 25°C inoculated with *A. niger* spores, having a titre between 10^4 - 10^5 , colony forming units/gram sample were found. At 8°C the wild-type strain was surviving well in soil as well as in surface water, whereas in waste water it was decreased beneath the detection level.

In the surface water environment the industrial strain decreased rapidly (within 2 months) below the detection level, whereas in soil instabilisation occurred when inoculated with a low (10^2 /g soil) titre.

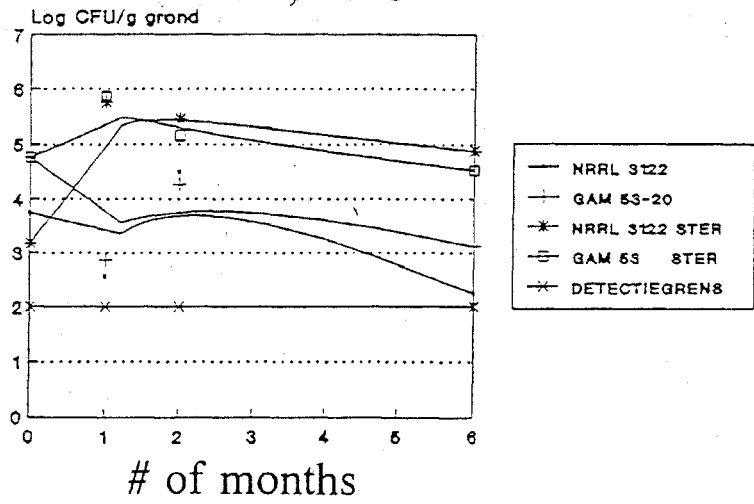
In natural environments and in the presence of competitive micro-organisms surviving *Aspergillus niger* strains were found after 6 month only in soil samples at 25°C, inoculated with at least 10^3 spores/g. In both aqueous environments at 8°C and 25°C and in soil of 8°C a fast decline of surviving strains was observed: within three months and often even faster titres decreased below the detection level.

During optimal conditions (when there is no competition) *Aspergillus niger* survives well in different environments, and the industrial strain usually less compared to the wild-type. Under natural condition *Aspergillus niger* survives only in soil at 25°C (after 6 month titre is decreased only 50 times). In the other environments under different conditions both tested *Aspergillus* strains were not detectable any more within 1 to 3 months, showing that the industrial strain *A. niger* GAM-53 is biological restricted with respect to growth and environmental surviving.

Incubation of *A. niger* mycelium in soil, 8°C

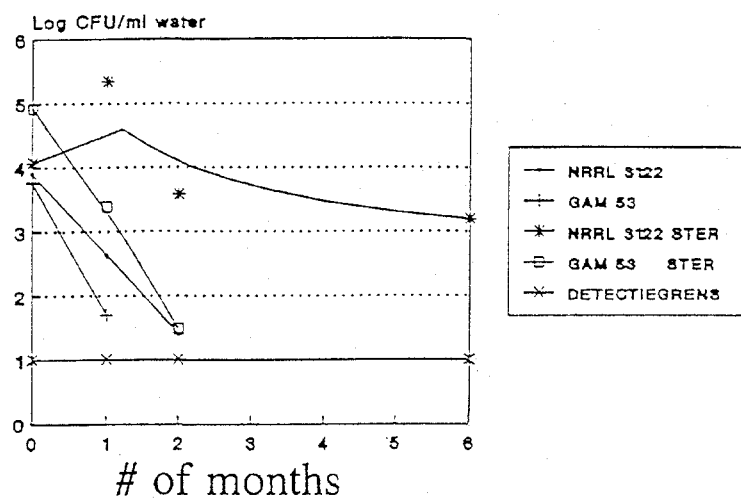


Incubation of *A. niger* mycelium in soil, 25°C

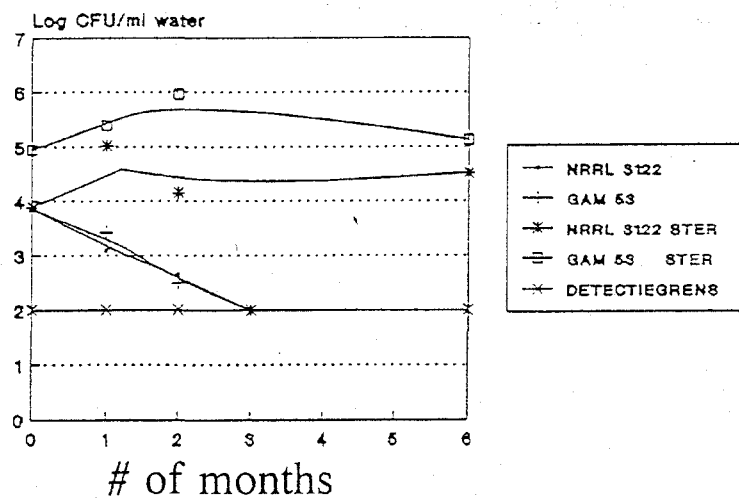


Surviving of *Aspergillus niger* I

Incubation of *A. niger* mycelium in surface water, 8°C

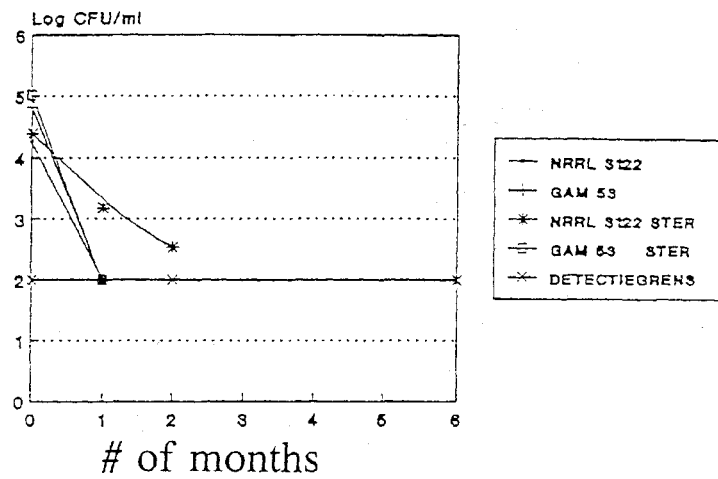


Incubation of *A. niger* mycelium in surface water, 25°C

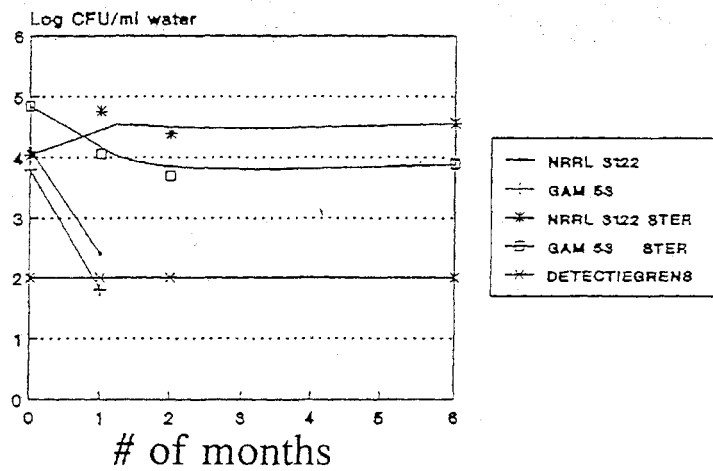


Surviving of *Aspergillus niger* II

Incubation of *A. niger* mycelium
in waste water, 8°C



Incubation of *A. niger* mycelium
in waste water, 25°C



Surviving of *Aspergillus niger* III

Annex 2.4.2

VROM Ministry of Housing
Spatial Planning and the Environment

> Return address: P.O. Box 1, 3720 BA Bilthoven. RIVM/SEC, BGGO

DSM Anti-Infectives B.V.
Attn.: Dr. P.W.M. van Dijck, DSM Strain Director
Internal post-code 530-0375
P.O. Box 1
2600 MA DELFT

**Directorate-General for Environmental
Protection**
Direction Riskpolicy

RIVM/SEC/Bureau GGO
Anthonie van Leeuwenhoeklaan 9
P.O. Box 1
3720 BA Bilthoven

Contactperson
Bureau GGO
Tel: 030-2742793
Fax: 030-2744401
bggo@rivm.nl
www.vrom.nl/ggo-vergunningverlening

Decree new application : EVO 09-011

Date 01-12-2009	Reference EVO 09-011.bes	Attachments
Your letter 16-10-2009	Your reference PWMvD/REG#55713	Copies to: Commission Genetic Modification (COGEM)

Dear Board of Management,

Herewith I send you a permit based on the Decree Genetically Modified Organisms Act
Environmentally dangerous substances (Official Journal 1993, 435), with the number EVO
09-011.

Sincerely,

Dr. H.C.M. van den Akker
Bureau GGO (Bureau for GMO Affairs)

Directorate-General for Environmental Protection
Direction Riskpolicy
Department Radiation, Nuclear and Biosafety

Rijnstraat 8
P.O. Box 30945
2500GX The Hague
Internal postcode 645

www.vrom.nl

Decree
DGM/SAS EVO 09-011/00

The Minister of Housing, Spatial Planning and the Environment (VROM),

Having read the application of DSM Anti-Infectives B.V., in Delft, 16-10-2009, reference: PWMvD/REG#55713, and the supplementary information of 16-11-2009, reference: PWMvD/REG#55802,

Based on article 2 of the Decree genetically modified organisms environmental protection (Official Journal 1993, 435),

Considering the provisions of the Regulation Genetically Modified Organisms (Official Journal 1998, 108), in particular article 3 of this Regulation,

Decides:

The strains *Aspergillus niger* TOL528-14 and the classically derived strains TOL-1 u/I TOL-100 belong to genetically modified organisms of group I with which activities of both category A and B may be carried out.

The Hague, 30-11-2009

The Minister of Housing, Spatial Planning and the Environment,
On his behalf, the Director-general of the State Institute for Public Health and the Environment
b.o. Head of the Office for Genetically modified organisms (Bureau GGO).

H.P. de Wijs

Interested parties may oppose to this decision, within six weeks after it has been send, based on the General Administrative Law, by submitting a motivated petition to the State Secretary of Housing, Spatial Planning and the Environment. The petition has to be addressed to: The Ministry of VROM attn. RIVM/SEC/Bureau GGO, P.O. Box 1, 3720BA Bilthoven. The petition has to bear a date and a name and address. It must be shown clearly why the decision is petitioned and when possible a copy of the decision should be included.



Ministerie van Volkshuisvesting,
Ruimtelijke Ordening en
Milieubeheer

> Retouradres: Postbus 1, 3720 BA Bilthoven, RIVM/SEC, BGGO

DSM Anti-Infectives B.V.
T.a.v. dr. P.W.M. van Dijk, DSM Strain Director
postpunt 530-0375
Postbus 1
2600 AM Delft

**Directoraat-Generaal
Milieu**
Directie Risicobeeld

RIVM/SEC/Bureau GGO
Antonie van
Leeuwenhoeklaan 9
Postbus 1
3720 BA Bilthoven

Contactpersoon
Bureau GGO

Telefoon 030 - 274 2793
Fax 030 - 274 4401
bggo@rivm.nl
www.vrom.nl/ggo-
vergunningverlening

Beschikking nieuwe aanvraag: EVO 09-011

Datum

01 DEC. 2009

Kenmerk

EVO 09-011.bes

Bijlagen

Uw brief

16-10-2009

Uw kenmerk

PWMvD/REG#55713

Afschrift aan

Commissie Genetische Modificatie

Geachte Directie,

Hierbij zend ik u een verklaring op grond van het Besluit genetisch gemodificeerde organismen milieubeheer (Stb. 1993, 435), met nummer DGM/RB EVO 09-011.

Hoogachtend,

(b) (6)

dr. H.C.M. van den Akker
Bureau GGO



Directoraat-Generaal Milieu
Directie Risicobeleid
Afdeling Straling, Nucleaire en Bioveiligheid

Rijnstraat 8
Postbus 30945
2500 GX Den Haag
Interne postcode 645

www.vrom.nl



beschikking

DGM/RB EVO 09-011/00

De Minister van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer,

Gelezen de aanvraag van DSM Anti-Infectives B.V., te Delft, van 16-10-2009, kenmerk: PWMvD/REG#55713 en de aanvullende informatie van 16-11-2009, kenmerk: PWMvD/REG#55802,

Gelet op artikel 2 van het Besluit genetisch gemodificeerde organismen milieubeheer (Staatsblad 1993, 435),

Overwegende,
het bepaalde in de Regeling genetisch gemodificeerde organismen (Staatscourant 1998, 108), in het bijzonder artikel 3 van de Regeling,

Besluit:
De stammen *Aspergillus niger* TOL528-14 en de daarvan klassiek afgeleide stammen *Aspergillus niger* TOL-1 t/m 100 behoren tot genetisch gemodificeerde organismen van groep I waarmee activiteiten van categorie A en B mogen worden uitgevoerd.

Den Haag, 30-11-2009
de Minister van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer,
voor deze:
de directeur-generaal van het Rijksinstituut voor Volksgezondheid en Milieu,
op last:
het hoofd van het Bureau Genetisch gemodificeerde organismen

(b) (6)

H. P. de Wijs

Tegen deze beschikking kunnen belanghebbenden binnen zes weken na verzending van de beschikking op grond van de Algemene wet bestuursrecht een gemotiveerd bezwaarschrift indienen bij de Minister van



Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer. Het bezwaarschrift moet gezonden worden aan:

De Minister van VROM

t.a.v RIVM/SEC/Bureau GGO

postbus 1

3720 BA Bilthoven.

Een bezwaarschrift moet van een datum en een naam en adres voorzien zijn. Er moet duidelijk worden aangegeven waarom tegen de beschikking, onder vermelding van het beschikingsnummer, bezwaar wordt aangetekend.

Annex 2.4.3

FRENCH REPUBLIC
Ministry of Ecology
And of sustainable Development

Scientific Committee
of the High Council of Biotechnologies

Paris, March 2, 2010

The Chairman of the scientific Committee

NOTIFICATION 10/516

The Scientific Committee of the High Council of Biotechnologies has been seized of a notification request registered under the number 10/516. This dossier submitted by the Company DSM Food Specialties concerns the use of *Aspergillus niger* strains containing few copies of the enzyme gene for the industrial production of the enzyme acid lactase.

The Scientific Committee of the High Council of Biotechnologies, after having proceeded to the examination of this dossier during its meeting of **23 February, 2010**, considers that these operations can be classified as follows:

Class 1 – Group I – containment level L1

Note : the containment L1 mandatory implies the inactivation of the effluents and of the wastes

Jean-Christophe PAGES



COMITE SCIENTIFIQUE
DU HAUT CONSEIL DES BIOTECHNOLOGIES

Fait à Paris, le 2 mars 2010

Le Président du Comité scientifique

AVIS 10/516

Le Comité Scientifique du Haut Conseil des Biotechnologies a été saisi d'une demande d'avis enregistrée sous le numéro 10/516. Cette demande déposée par la Société **DSM Food Specialties France SAS** porte sur la **production industrielle de l'enzyme lactase acide par des souches d'*Aspergillus niger* contenant plusieurs copies du gène de l'enzyme.**

Le Comité Scientifique du Haut Conseil des Biotechnologies, après avoir procédé à l'examen de ce dossier lors de sa séance du **23 février 2010**, estime que ces opérations peuvent être classées comme suit :

Classe I – groupe I – confinement L1

Note : Le confinement L1 implique obligatoirement l'inactivation des effluents et des déchets.

(b) (6)

Jean-Christophe PAGÈS

Vous avez la possibilité de contester la décision par deux voies distinctes :

- Un recours administratif exercé dans le délai de deux mois à compter de la réception de cette décision auprès de la ministre de l'enseignement supérieur et de la recherche.
- Un recours contentieux exercé dans le délai de deux mois de cette décision ou à compter du rejet de votre recours administratif, auprès du tribunal administratif de Paris.

Annex 3.2.1

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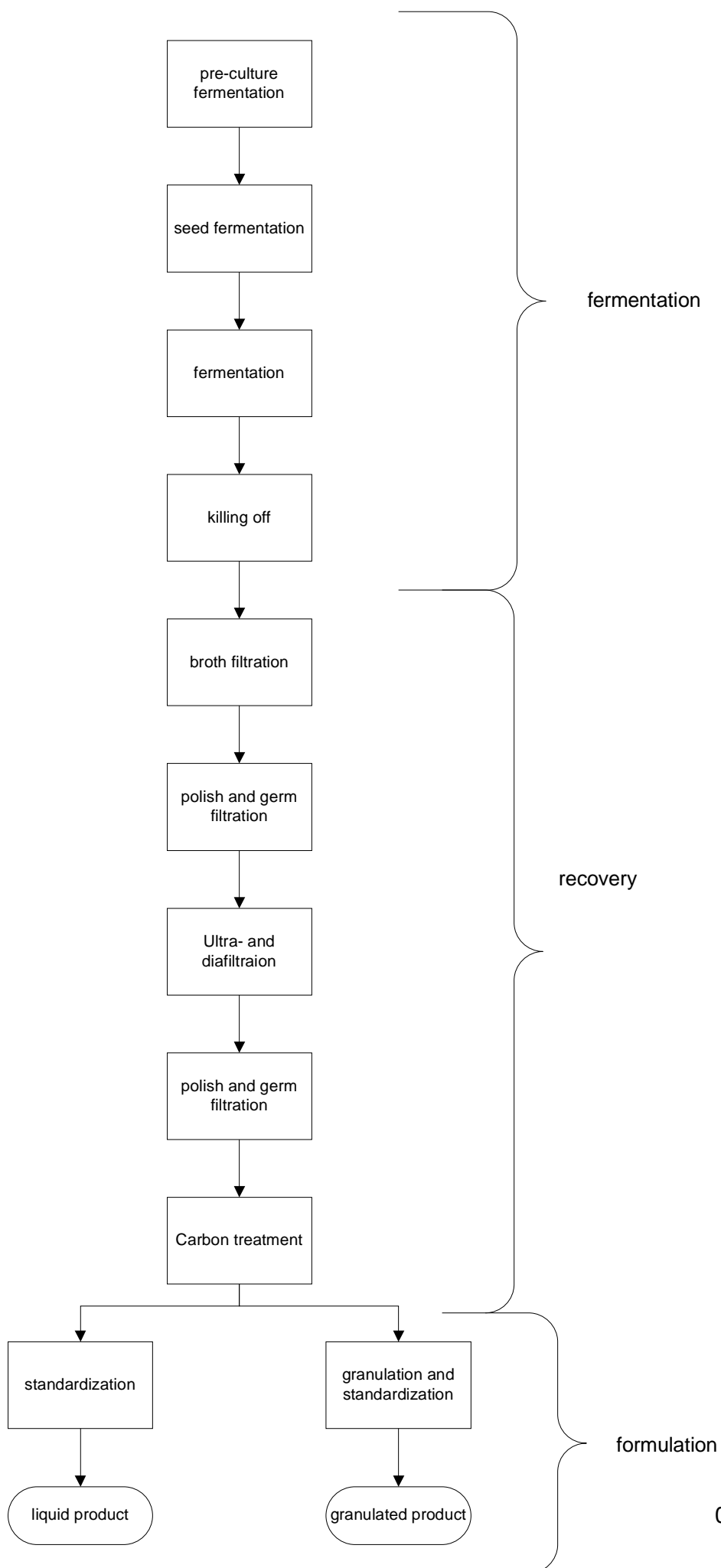
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RNLTTGVYTD  TSDLAVTPLI  GDSPGSFFVV  RHTDYSSQES  TSYKLKLPST
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KVLVLYGGPK  EHHELAIASK  SNVTIEGSD  SGIVSTRKGS  SVIIGWDVSS
TRRIVQVGD  RVFLLDNRSA  YNYWVPELPT  EGTSPGFSTS  KTTASSIIVK
AGYLLRGAHL  DGADLHLTAD  FNATTPIEVI  GAPTGAKNLF  VNGEKASHTV
DKNGIWSSEV  KYAAPEIKLP  GLKDLWDKYL  DTLPEIKSSY  DDSAWVSADL
PKTKNTHRPL  DTPTSLYSSD  YGFHTGYLIY  RGHFVANGKE  SEFFIRTQGG
SAFGSSVWLN  ETYLGSWTGA  DYAMDGNSTY  KLSQLESGKN  YVITVVIDNL
GLDENWTVGE  ETMKNPRGIL  SYKLSGQDAS  AITWKLTGNL  GGEDYQDKVR
GPLNEGGLYA  ERQGFHQPP  PSESWESESP  LEGLSKPGIG  FYTAQFDLNL
PKGWDVPLYF  NFGNNTQAAR  AQLYVNGYQY  GKFTGNVGPQ  TSFPVPEGIL
NYRGNTNYVAL  SLWALESDGA  KLGSFELSIT  TPVLTGYGNV  ESPEQPKYEQ
RKGAY

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Amino acid sequence of the acid lactase precursor protein.

The precursor protein is transported into the endoplasmic reticulum of *A. niger* and, ultimately, into the extracellular medium; the signal peptidase cleavage site is most likely located after position 18. The mature acid lactase protein most likely runs from position 19 (underlined) until position 1005 and has a molecular mass of ~97 kDa after secretion as determined on SDS-PAGE

Annex 4.1.1



Annex 7.1.1

Pages 000097-000106 have been removed in accordance with copyright laws. The removed reference is:

Schuster, E., Dunn-Coleman, N., Frisvad, J.C., Dijck, P.W.M van, *On the Safety of Aspergillus niger – a Review*, Appl. Microbiol. Biotechnol. 59:426-435 (2002)

Annex 7.1.2



ENZYMES DERIVED FROM ASPERGILLIS NIGER

EXPLANATION

A. niger is a contaminant of food and was not considered in the same light as those organisms regarded as normal constituents of food. It is necessary to show that the strains used in enzyme preparations do not produce mycotoxins.

Microbial carbohydrases prepared from some varieties of *A. niger* were evaluated at the fifteenth meeting of the Committee, at which time a temporary ADI "not limited" was established (Annex 1, reference 26). A toxicological monograph was prepared (Annex 1, reference 27). An adequate 90-day study in rats was requested. Since the previous evaluation, additional data have become available on a number of carbohydrases, which are summarized and discussed in the following monograph. These enzymes were considered by the Committee to encompass the carbohydrases previously considered. The previously published monograph has been expanded and reproduced in its entirety below.

AMYLOGLUCOSIDASES (E.C. 3.2.1.3)

BIOLOGICAL DATA

Biochemical aspects

No information available.

Toxicological studies

Special studies on aflatoxin-related effects

Ducklings

Four groups of 5 ducklings received in their diet 0, 1, 5, or 10% enzyme preparation for 29 days. Growth, feed consumption, survival, behaviour, and mean liver weights were comparable, in all groups. No gross or histopathological lesions of the liver were seen (FDRL, 1963a).

Four groups of 5 ducklings received in their diet 0, 1, 5, or 10% enzyme preparation for 29 days. Growth, feed consumption, survival, behaviour, and development were comparable in all groups. No gross liver lesions were seen at autopsy and mean liver weights of treated animals were similar to those of controls. Histopathology of the livers was normal. No toxic elements were noted (FDRL, 1963b).

Acute toxicity¹

Species	Route	LD ₅₀ (mg/kg b.w.)	Reference
Mouse	oral	> 3,200	Hunt & Garvin, 1963
		> 4,000	Hunt & Garvin, 1971
		> 3,200	Willard & Garvin, 1968
		> 4,000	Garvin et al., 1966
Rat	oral	10,000	Gray, 1960
		31,600	Kay & Calendra, 1962
		> 3,200	Willard & Garvin, 1968
		> 4,000	Garvin et al., 1966
		12,500 - 20,000	Kapiszka & Hartnage, 1978
Rabbit	oral	> 4,000	Garvin et al., 1966
Dog	oral	> 4,000	Garvin et al., 1966

¹ These data were obtained with several different commercial enzyme preparations.

Short-term studies

Rats

Three groups of 10 male rats received 0, 0.5, or 5% enzyme preparation in their diets for 30 days. No adverse effects related to treatment were observed regarding growth, appearance, behaviour, survival, food consumption, haematology, organ weights, or gross pathology (Garvin et al., 1966).

Two groups of 10 male and 10 female rats received either 0 or 5% enzyme preparation in their diets daily for 91 days. No differences from controls were observed regarding appearance, behaviour, survival, weight gain, haematology, organ weights, or gross pathology (Garvin & Merubia, 1959).

Two groups of 10 male and 10 female ARS Sprague-Dawley rats were fed diets containing 5 or 10% of the test enzyme preparation (equivalent to 3.5 or 7 g enzyme preparation/kg b.w./day) for 90 to 94 days. A control group of 20 male and 20 female rats were maintained on the diet alone. No signs of toxicity were observed during the test period. Body-weight gain and food consumption were similar between test and control groups. Differential blood counts were within the normal range at weeks 4 and 8 of the study in both test and control animals. At the end of the study serum clinical chemistry parameters, organ weight analyses, and gross and microscopic pathology showed no compound-related effects (Garvin et al., 1972).

Long-term studies

No information available.

Observations in man

No information available.

COMMENTS

Several short-term feeding studies in rats on amyloglucosidase preparations from *A. niger* have been performed. One study, in which the preparation was fed at up to 10% of the diet, was considered to be acceptable by current standards. No compound-related effects were observed in this study or in duckling tests that were performed to investigate potential aflatoxin-related effects.

The evaluations by the Committee of the carbohydrates and the protease from *A. niger* are summarized at the end of this section.

REFERENCES

- FDRL (1963a). Unpublished report No. 84600e. Submitted to WHO by Laboratories, Inc., Elkhart, IN, USA.
- FDRL (1963b). Unpublished report No. 84600f. Submitted to WHO by Laboratories, Inc., Elkhart, IN, USA.
- Garvin, P.J. & Merubia, J. (1959). Unpublished report. Submitted to WHO by Baxter Laboratories, Inc.
- Garvin, P.J., Willard, R., Merubia, J., Huszar, B., Chin, E., & Gilbert, C. (1966). Unpublished report. Submitted to WHO by Baxter Laboratories, Inc.
- Garvin, P.J., Ganote, C.E., Merubia, J., Delahany, E., Bowers, S., Varnado, A., Jordan, L., Harley, G., DeSmet, C., & Porth, J. (1972). Unpublished report from Travenol Laboratories, Inc., Morton Grove, IL, USA. Submitted to WHO by Gist-brocades NV, Delft, Holland.
- Gray, E.H. (1960). Unpublished report. Submitted to WHO by Miles Laboratories, Inc., Elkhart, IN, USA.
- Hunt, R.F. & Garvin, P.J. (1963). Unpublished report. Submitted to WHO by Baxter Laboratories, Inc.
- Hunt, R.F. & Garvin, P.J. (1971). Unpublished report. Submitted to WHO by Travenol Laboratories, Inc., Morton Grove, IL, USA.
- Kapiszka, E.L. & Hartnage, R.E. (1978). The acute oral toxicity of Diazyme concentrate and Diazyme 325 in the rat. Unpublished report No. 16 from Miles Laboratories, Inc., Elkhart, IN, USA. Submitted to WHO by Miles Laboratories, Inc., Elkhart, IN, USA.
- Kay, J.H. & Calendra, J.C. (1962). Unpublished report. Submitted to WHO by Miles Laboratories, Inc., Elkhart, IN, USA.
- Willard, R. & Garvin, P.J. (1968). Unpublished report. Submitted to WHO by Travenol Laboratories, Inc., Morton Grove, IL, USA.
- β -GLUCANASE (E.C. 3.2.1.6)

BIOLOGICAL DATA

Biochemical aspects

No information available.

Toxicological studies

(The TOS of the enzyme preparation used for toxicity studies was 49%).

Special Studies on mutagenicity

The enzyme preparation was tested for mutagenic activity using 5 strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537, and TA1538 both with and without metabolic activation (S-9 fraction). The preparation was not mutagenic or toxic at concentrations up to 40 mg/ml (McConville, 1980).

A cytogenic bone marrow study was performed using adult male Chinese hamsters. Groups of adult male hamsters received up to 5000 mg/kg b.w./day of the enzyme preparation for 5 consecutive days. Treatment did not result in an increased frequency of chromosomal aberrations in bone marrow (McGregor & Willins, 1981).

Acute toxicity

Species	Route	Sex	LD ₅₀ (ml/kg b.w.)	Reference
Mouse (NMRI)	oral	M & F	30	Novo, 1978a
Rat (Wistar)	oral	-	28.1	Novo, 1978b

Short-term studies

Rats

Three groups, each containing 5 male and 5 female Wistar/Mol SPF rats, were dosed orally by gavage once a day for 14 days with enzyme preparation at dose levels equivalent to 2.5, 5.0, or 10 ml/kg b.w. No clinical changes were observed. Body-weight gains of test and control animals were similar. At termination of the study, measurements of organ weights showed no compound-related effects (Novo, 1978c).

In another study, 4 groups, each containing 15 male and 15 female Wistar/Mol SPF rats, were dosed by gavage once a day for 90 days with enzyme preparation at dose levels equivalent to 0, 2.5, 5.0, or 10 ml/kg b.w. Deaths, primarily in the high-dose group, appeared to be related to injury during dosing. No clinical signs were observed in the other test animals. Male rats in the high-dose group showed decreased weight gain and marked decrease in food intake. Haematology studies showed increased platelet counts and decreased clotting times

in the high-dose group at week 6, but this effect was not apparent at week 12. No other effects were reported. Clinical chemistry and urinalysis values at weeks 6 and 12 were within the normal range. At termination of the study, organ weight analysis showed a marked increase in relative weights of the spleen and testes of the males in the high-dose group. Gross and histopathological examination of the principal organs and tissues showed no compound-related effects (Perry *et al.*, 1979).

Dogs

Three groups, each containing one male and one female beagle dog, received single doses of 5, 10, or 15 ml/kg b.w. of the enzyme preparation over a 4-day period. Following a 7-day observation period the dogs were sacrificed and subjected to macroscopic post-mortem examination. No compound-related effects were observed, with the exception of vomiting during the first 4 days of the study. In another study, dogs were administered consecutive doses of 15 ml/kg b.w./day for 9 days, and 10 ml/kg b.w./day for 5 days. No deaths occurred during the course of the study. The only clinical sign noted was excessive salivation and emesis shortly after dosing. Body weights, electrocardiograms, haematological parameters, blood serum chemistry, organ weights, gross pathology, and histopathology showed no compound-related effects (Osborne *et al.*, 1978).

In another study, three groups, each containing 3 male and 3 female beagle dogs, were dosed with the enzyme preparation by gavage once a day, seven days a week, for 13 weeks, at dose levels equivalent to 2, 5, or 9 ml/kg b.w./day. Two dogs in the high-dose group died during the course of the study, which the authors concluded was due to respiratory distress as a result of foreign material in the lungs. Vomiting was reported after dosing in the high-dose group. Haematological parameters at weeks 6 and 12 were within normal limits, with the exception of a significant increase in WBC count, specifically in the group mean neutrophil counts, in the high-dose group. Clinical chemistry values were within the normal range at weeks 8 and 12, with the exception of slight increases in blood glucose and cholesterol in the high-dose group. Urinalysis showed no compound-related effects. At termination of the study, organ-weight analyses and gross and histopathological examination of the principal organs and tissues showed no compound-related effects (Greenough *et al.*, 1980).

Long-term studies

No information available.

Observations in man

No information available.

COMMENTS

This enzyme preparation was not genotoxic in microbial or in mammalian test systems. Short-term studies in rats and dogs resulted in no observed compound-related effects at levels up to 5 ml/kg b.w./day of enzyme preparation.

The evaluations by the Committee of the carbohydrases and the protease from *A. niger* are summarized at the end of this section.

REFERENCES

Greenough, R.J., Brown, J.C., Brown, M.G., Cowie, J.R., Maule, W.J., & Atken, R. (1980). β -Glucanase 13 week oral toxicity study in dogs. Unpublished report No. 1630 from Inveresk Research International, Musselburgh, Scotland. Submitted to WHO by Novo Industri A/S, Bagsvaerd, Denmark.

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HEMI-CELLULASE

BIOLOGICAL DATA

Biochemical aspects

No information available.

Toxicological studies

Special studies on mutagenicity

The enzyme preparation was tested for mutagenic activity using *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 both with and without metabolic activation (S-9 fraction). The test substance was not mutagenic or toxic at concentrations up to 5 mg/plate (Clausen & Kaufman, 1983).

In an *in vitro* cytogenetic test using CHO-K1 cells, both with and without metabolic activation (S-9 fraction), the enzyme preparation at test levels up to 2.5 mg (dry wt)/ml did not induce chromosomal aberrations (Skovbro, 1984).

Acute toxicity

No information available.

Short-term studies

Rats

Four groups, each containing 5 male and 5 female Wistar MOL/W rats, were dosed by gavage once a day for 90 days with the enzyme preparation at doses equivalent to 0, 100, 333, or 1000 mg/kg b.w./day. No significant clinical changes were observed. Body-weight gain and food intake were similar among test and control animals. Haematologic and clinical chemistry measurements at termination of the study were within normal ranges. Post-mortem examinations, measurements of organ weights, and histopathology showed no compound-related effects. Slight increases in kidney and adrenal weights in the mid-dose group were not associated with histopathological effects, and did not show a dose response (Kallensen, 1982).

Long-term studies

No information available.

Observations in man

No information available.

COMMENTS

This enzyme preparation was not genotoxic in microbial or in mammalian test systems. In a limited 90-day study in rats, no effects were observed at the highest dose administered (1 g/kg b.w./day). This enzyme preparation contained high levels of pectinase. The pectinase enzyme preparation summarized below may be identical to this hemi-cellulase preparation, which provides added assurance of the safety of this preparation.

The evaluations by the Committee of the carbohydrases and the protease from *A. niger* are summarized at the end of this section.

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PECTINASE (E.C. 3.1.1.11; 3.2.1.15; 4.2.2.10)

BIOLOGICAL DATA

Biochemical aspects

No information available.

Toxicological studies (The TOS of the commercial preparation is approximately 5%).

Acute toxicity

Species	Route	LD ₅₀ (ml/kg b.w.)	Reference
Rat	oral	18.8-22.1	Porter & Hartnagel, 1979

Short-term studies

Rats

Two groups of 10 male and 10 female ARS Sprague-Dawley rats were fed diets containing 5 or 10% of the test enzyme preparation (equivalent to 3.5 or 7 g of the enzyme preparation/kg b.w./day), for 90 to 94 days. A control group of 20 male and 20 female rats was maintained on the diet alone. No signs of toxicity were observed during the test period. Body-weight gain and food consumption were similar among test and control groups. Differential blood counts at weeks 4 and 8 of the study were within the normal range in test and control animals. At the end of the study serum clinical chemistry analyses, organ weight analyses, and gross and microscopic pathology showed no compound-related effects (Garvin *et al.*, 1972).

Long-term studies

No information available.

Observations in man

No information available.

COMMENTS

In a short-term study in rats, no adverse effects were observed at dietary levels of the enzyme preparation up to the equivalent of 7 mg/kg b.w./day. This enzyme preparation may be identical to the hemi-cellulase preparation summarized above. The hemi-cellulase enzyme preparation summarized above also contained high levels of pectinase, which provides added assurance of the safety of this preparation.

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- Porter, M.C. & Hartnagel R.E. (1979). The acute oral toxicity of a new pectinase product in the rat. Unpublished report No. 11 from Miles Laboratories, Inc., Elkhart, IN, USA. Submitted to WHO by Enzyme Technical Association, Washington, DC, USA.

PROTEASE

No information available.

GENERAL COMMENTS ON ENZYMES FROM *A. NIGER*

Aspergillus niger is a contaminant of food. Although there may be possible strain differences in *A. niger*, and different cultural conditions might be used to prepare the various enzymes, the available toxicity data, which consist primarily of short-term feeding studies in rats and some studies in dogs, show that all the enzyme preparations tested were of a very low order of toxicity. The enzyme preparations tested were non-mutagenic in bacterial and mammalian cell systems. Studies on some strains of *A. niger* used to prepare carbohydrases showed no aflatoxin or related substance production. These studies provide the basis for evaluating the safety of enzyme preparations derived from *A. niger*. It was also noted that the enzyme preparations tested exhibit a number of enzyme activities, in addition to the major enzyme activity. Thus, there may be considerable overlap of the enzyme activities of the different enzyme preparations so that safety data from each preparation provides additional assurance of safety for the whole group of enzymes.

Since the enzyme preparations tested were of different activities and forms, and most of the organic materials in the preparations are not the enzyme *per se*, the numerical ADI is expressed in terms of total organic solids (TOS) (see introduction to enzyme preparations section).

EVALUATION

Level causing no toxicological effect

All enzyme preparations tested showed no-observed-effect levels greater than 100 mg TOS/kg b.w./day in 90-day studies in rats.

Estimate of acceptable daily intake

0-1 mg TOS/kg b.w. for each of the enzyme preparations.

See Also:

Toxicological Abbreviations

Annex 7.1.3

DATE: September 14, 1988

SUBJECT: Proposed Acceptable Daily Intake (ADI) Levels For
Enzymes From Organisms Not Commonly Considered To Be
Constituents of Food

FROM: J. W. Bennett, Ph.D.
Professor of Biology
Tulane University

I have read the report of the Joint FAO/WHO Expert Committee of
Food Additives 31st meeting, Geneva, February 16-25, 1987

This report reiterates a conclusion reached at an earlier meeting
of JECFA that an acceptable daily intake (ADI) should be estab-
lished for certain enzyme preparations derived from microorgan-
isms not normally used as food, or for enzyme preparations not
removed from the food products to which they are added. This
conclusion is based, in part, on the notion "that source organ-
isms may produce toxins under certain conditions of growth"

Neither the name of the putative toxins, nor the name of the
organisms implicated as toxin producers, was given in the report.

I would assume that the "offending" species are Aspergillus
niger, Trichoderma harzianum, Trichoderma reesei, Penicillium
funiculosum, and Aspergillus alliaceus since these are the
producing organisms for enzymes for which the Joint FAO/WHO
Expert Committee seeks to establish ADI's. Since none of these

species has been documented to produce mycotoxin in industrial applications, my comments below pertain more toward hypothetical situations, involving the introduction of new producing strains in the future, than to the species for which the ADI's are currently proposed. Based on the lack of documented evidence of toxin production in industrial settings, it is my opinion, that there is no reason to establish ADI's for the enzymes or species listed in the Table (ICS/87.13 Page 3 of the Summary and Conclusions of the Joint FAO/WHO Expert Committee on Food Additives entitled "Acceptable daily intakes, other toxicology information, and information on specifications" (Part A. Food additives, Enzyme preparations)).

Before speaking to the questions raised by the report of the Joint FAO/WHO Expert Committee of Food Additives, it is important that certain terms be defined. Selected references, cited by author and date, are included in the text below. A bibliography is affixed at the end of the report.

Mycotoxins are fungal secondary metabolites that evoke a toxic response when introduced in low concentration to higher vertebrates, and other animals, by a natural route. Pharmacologically active fungal products such as antibiotics (which are toxic to bacteria and ethanol which is toxic to

animals but only in high concentration) are excluded from this definition (Bennett, 1987

Secondary metabolites are low molecular weight compounds of enormous chemical diversity and restricted taxonomic distribution that are normally synthesized after active growth has ceased. Secondary metabolites are biosynthesized from small precursor molecules e.g., acetate, malonate, isoprene, amino acids) via a series of enzymatic conversions. Production of secondary metabolites is both species and strain specific (Bennett & Ciegler, 1983).

Species are basic taxonomic units. Fungal species are named in accordance with the rules governed by the International Code of Botanical Nomenclature. The term "strain" derives from the International Code of Nomenclature of Bacteria. A strain constitutes the descendants of a single isolation in pure culture, sometimes showing marked differences in economic significance from other strains or isolations. Strain is analogous to "clone" in the International Code of Botanical Nomenclature (Jeffrey 1977; Bennett, 1985

The ability to produce a mycotoxin or other secondary metabolite is a characteristic of a species. Within the species different strains may vary in their biosynthetic potential: some strains may be high producers, some may be low producers, some

may be non-producers. The most common variant is the non-producer

Having defined the relevant terms, it is now possible to address certain issues raised by the report of the Joint FAO/WHO Expert Committee. The commentary below is organized as a series of questions and answers.

1. Do non-toxicogenic species of fungi develop strains that produce detectable levels of mycotoxins? Is the fact that mycotoxins are secondary metabolites relevant to this questions?

No. Non-toxicogenic species of fungi do not become toxicogenic. However, the reverse is true. It is quite easy to isolate non-toxicogenic mutants and variants as clones "strains" from toxicogenic species

The fact that mycotoxins are secondary metabolites is very relevant. Unlike enzymes, which are direct gene products synthesized directly from a structural gene via a series of RNA and amino acid intermediates, secondary metabolites are the result of numerous biosynthetic steps, each step enzymatically catalyzed. In most cases we do not know the exact number of steps in a biosynthetic pathway for a given secondary metabolite. Therefore, we do not know the number of genes required to encode for the enzymes of the pathway. However, all secondary metabolites are biosynthesized by

multistep pathways with many genes and many enzymes involved.

2. Can conventional mutation (by mutagens or UV) or changes in medium or growth conditions cause a demonstrated non-toxin producer to begin producing toxins?

No. "You can't get something from nothing". Organisms which lack the structural genes for the enzymes of a mycotoxin pathway cannot be turned into toxin producers by simple mutation or changes in environmental parameters. In order for a non-toxicogenic species to become toxicogenic it would have to acquire the genes for an entire biosynthetic pathway.

A basic precept from genetics is analogous here: Deletions do not revert. Put another way, the absence of genetic material cannot mutate. Nor can it be expressed. Again note that the reverse is possible. Toxicogenic species may mutate to non-toxicogenic strains; and under certain growth conditions, toxicogenic strains may not express the genetic material for toxin production.

3. Since enzymes are primary metabolites which are ordinarily produced in the logarithmic phase of growth, what is the likelihood that mycotoxins, which are secondary metabolites, would be co-produced with the enzymes?

Usually there would be no co-production of secondary metabolites with the enzymes harvested during growth phase. Modern fermentation technology relies heavily on submerged cultures for growing production strains of fungi. Commercial enzymes are usually isolated from actively growing cultures. Because filamentous fungi grow in the form of thread-like hyphal cells, this early phase of growth, roughly analogous to logarithmic growth in single-celled organisms, has been given a special name: "trophophase". Similarly, in the jargon of fungal physiology, the period after active growth has ceased is called "idiophase". Idiophase is roughly analogous to lag phase or stationary phase for single-celled organisms. Most of the time, no secondary metabolites are produced during trophophase (Turner, 1971, pp. 18-20). Since this early growth phase is the phase during which most commercial enzymes are harvested, even in toxicogenic species it is possible to avoid accumulation of toxins by early harvesting of the fermentation cultures.

It is also relevant that the majority of mycotoxins are only sparingly soluble in water. Chemical separations of most mycotoxins use nonpolar solvents (Cole and Cox, 1981

Enzymes, on the other hand, are isolated with water and other polar solvents.

4. It is common practice for industry to test organisms for toxicogenicity and pathogenicity and products for non-specific toxicity before introducing them into commercial production and to test specifically for a toxin known to be associated with a given species. Is it appropriate for JECFA to impose testing for aflatoxin B1, ochratoxin A, sterigmatocystin, T-2 toxin and zearalenone in all fungal-derived enzyme preparations?

Once a producing species has been demonstrated as non-toxicogenic, it is a waste of time and money to test each lot of a commercial preparation for toxin production

If a species lacks the genetic material to biosynthesize a toxin, it will remain non-toxicogenic. Biosynthetic capacity is part of a species definition

A clumsy but colorful analogy could be drawn from the animal world. It would not make sense to test chickens and their eggs for milk production; nor would it be logical to assay cows and milk for the presence of feathers. Some vertebrates make milk; some make feathers. However, just because an organism is a vertebrate does not mean it will make either of these substances. Similarly, although some species of fungi make aflatoxin or T-2 toxin, it does not make sense to test all fungal preparations for aflatoxin and T-2 toxin

Specifically, there is no reason to test Aspergillus niger, Penicillium funiculosum, Trichoderma harzianum or T. reesei for aflatoxin B₁, sterigmatocystin, ochratoxin, T-2 toxin or zearalenone. Since some strains of Aspergillus alliaceus are known ochratoxin producers, enzyme preparations from this species might be tested for this one toxin. It would not be necessary to test A. alliaceus preparations for aflatoxin B₁, sterigmatocystin, T-2 toxin, or zearalenone.

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Sep. 1988

THE OCCURRENCE AND SIGNIFICANCE
OF MYCOTOXINS

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THE OCCURENCE AND SIGNIFICANCE OF MYCOTOXINS

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1. Description of mycotoxins

A conservative estimate suggests that there are at least 100,000 species of fungi (Hawksworth, Sutton & Ainsworth, 1983) and many of these are able to produce one or more low molecular weight organic compounds known as secondary metabolites. These metabolites are a structurally diverse group of molecules (Turner & Aldridge, 1983) some of which have biological activity as antibiotics, phytotoxins and mycotoxins. The term mycotoxin is generally confined to those toxic metabolites produced by moulds growing on foods, animal feeds, or the raw materials and additives used in their manufacture.

The biological activity of mycotoxins is characterized by a toxic response when consumed by man or animals. Depending on the type of mycotoxin and animal species, even low concentrations of mycotoxins can create an acutely toxic, carcinogenic, oestrogenic or immuno-suppressive effect. A number of fungi producing macroscopic fruiting bodies (mushrooms and toadstools) also produce toxic metabolites and these are a hazard when such fruiting bodies are eaten. It is convenient to deal with these compounds separately and not include them as mycotixons.

2. Mycotoxins as natural contaminants in food

Of the several hundred known toxic mould metabolites (see Moreau, 1974; Wyllie & Morehouse, 1977; Watson, 1985) only about three dozen have been shown to occur as natural contaminants in food (Krogh, 1987). Table 1 lists the majority of these with the species of mould known to produce them.

A further selection of mycotoxins, such as the satratoxins, verrucarins, sporidesmins and slaframine, have been identified in animal feeds and fodders.

3. Ability of mycotoxin production depends on species as well as circumstances

Some mycotoxins are only produced by a limited number of strains of one or two species of fungi, whereas others may be produced by a large number of species. Thus the aflatoxins are only known to be produced by *Aspergillus flavus* and *A. parasiticus*, whereas ochratoxin is produced by several species of *Aspergillus* and *Penicillium*. It is not the case that species of mould traditionally used as constituents of food of producing mycotoxins.

Thus, *Aspergillus oryzae*, used extensively in the production of koji for the manufacture of a wide range of foods, is able to produce cyclopiazonic acid and β -nitro propionic acid, and *Penicillium roquefortii*, used in the manufacture of all the blue cheeses of the world, can produce PR-toxin, roquefortine and several other toxic metabolites. Because processes, and strain properties, are developed to optimise such qualities as biomass and industrial enzyme production (and are generally inversely related to those developed to optimize, or even permit, secondary metabolite formation), the production of koji and blue cheese is not associated with any known mycotoxin problem. In a sense, it is the process, rather than the organism, which is safe.

4. Species specific mycotoxins

Table 2 lists some of the secondary metabolites associated with species of mould used for the production of enzymes. Only *Aspergillus alliaceus* is known to produce one of the mycotoxins (ochratoxin) included in those routinely tested for using the method of Patterson and Roberts (i.e. aflatoxin B₁, ochratoxin A, sterigmatocystin, T-2 toxin and zearalenone). The major justification for looking for these mycotoxins in products from species not associated with their production must presumably be concern for carry over from contaminated raw materials, or a failure to maintain a pure culture during the manufacturing process.

5. Effect of mutations on mycotoxin production

The biosynthetic pathways leading to the production of mycotoxins are frequently complex involving many steps (Steyn, 1980). The majority, if not all, of these steps will involve an enzyme which in turn will be coded for by a gene. Thus many genes may be involved in the production of a particular mycotoxin. It is thus a common experience that the ability to produce a particular mycotoxin is readily lost during routine subculture of the producing strain. In fact, those who are trying to industrially produce secondary metabolites need to take special care to avoid this happening. It is also relatively easy to lose the capability of producing a mycotoxin by a deliberate programme of mutation. Since the chance to obtain a mutation defect in one of the many genes involved in mycotoxin synthesis is much higher than that of a mutation repair of one or more specific defects, the situation in which a non-toxigenic strain becomes toxigenic is far less common. Only one author (Benkhammar et al. (1985)) has reported obtaining cyclopiazonic acid producing mutants of *Aspergillus oryzae* by treating a non-toxigenic strain with a mutagenic N-nitroso-guanidine derivative.

6. Mycotoxin and enzyme production: likelihood of co-production

The growth and morphological and biochemical differentiation of filamentous fungi involve the sequential induction, formation and repression of many hundreds of enzymes, some of which are involved in the biosynthesis of mycotoxins.

However, the relatively small number of enzymes of industrial interest are usually associated with the earlier stages of vigorous growth and their production is directly growth related. This is in contrast to the production of mycotoxins most of which occurs during the later stages of development and their optimum production is often associated with some form of stress on growth processes.

In a limited study of strains of *Aspergillus flavus* and closely related species at the University of Surrey, it was found that an inverse correlation occurs between the ability of strains to produce aflatoxin and the ability to produce and secrete high levels of growth related catabolic enzymes such as amylases. Such observations are entirely compatible with the suggestion that *Aspergillus oryzae* and *A. sojae* are "domesticated" forms of *A. flavus* and *A. parasiticus* respectively (Wicklow, 1984).

7. Mycotoxins and enzyme purification: likelihood of co-isolation

The enzymes of particular interest in the food industry are globular proteins which are high molecular weight water soluble compounds in contrast to the low molecular weight secondary metabolites many of which are more soluble in organic solvents than in water.

If secondary metabolites, including mycotoxins, were present in the production liquors from which enzyme are obtained, it is highly probable that some stages in down stream processing, such as ultra filtration, will effect a partial removal.

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TABLE 1: Mycotoxins identified as natural contaminants in food associated commodities.

MYCOTOXIN	MAJOR PRODUCING SPECIES

AFLATOXINS	<u>Aspergillus flavus, A. parasiticus</u>
OCHRATOXIN	<u>Aspergillus ochraceus,</u> <u>Penicillium viridicatum</u>
CITRININ	<u>Penicillium citrinum</u>
PENICILLIC ACID	<u>Penicillium spp., Aspergillus spp.</u>
PATULIN	<u>Penicillium expansum,</u> <u>Aspergillus clavatus</u>
STERIGMATOCYSTIN	<u>Aspergillus versicolor</u>
MYCOPHENOLIC ACID	<u>Penicillium roquefortii</u>
PENITREM A	<u>Penicillium aurantiogriseum</u>
P R TOXIN	<u>Penicillium roquefortii</u>
VIOMELLEIN	<u>Aspergillus ochraceus</u> <u>Penicillium viridicatum</u>
CYTOCHALASIN E	<u>Aspergillus clavatus</u>
CITREOVIRIDIN	<u>Penicillium citreonigrum</u>
CYCLOPIAZONIC ACID	<u>Aspergillus flavus,</u> <u>Penicillium aurantiogriseum</u>
ROQUEFORTINE	<u>Penicillium roquefortii</u>
ISOFUMIGACLAVINE	<u>Penicillium roquefortii</u>
ZERALENONE	<u>Fusarium spp</u>
ZEARALENOL	<u>Fusarium spp</u>
TRICHOHECENES	<u>Fusarium spp</u>
MONILIFORMIN	<u>Fusarium spp</u>
TENUAZONIC ACID	<u>Alternaria spp</u>
ALTERNARIOL	<u>Alternaria spp</u>
ALTENUENE	<u>Alternaria spp</u>
ERGOT ALKALOIDS	<u>Claviceps spp</u>

TABLE 2: Examples of secondary metabolites reported to be produced by moulds used for the manufacture of enzymes.

MOULD SPECIES	METABOLITES
<u>Aspergillus alliaceus</u>	OCHRATOXINS A and B*
<u>Aspergillus niger</u>	RUBROFUSARIN B NIGERONE AURASPERONE NEOECHINULIN NIGRAGILLIN ASPERRUBROL
<u>Aspergillus oryzae</u>	B-NITROPROPIONIC ACID* MALTORYZINE* CYCLOPIAZONIC ACID* KOJIC ACID ORYZACIDIN ASPERGILLOMARASMIN
<u>Penicillium funiculosum</u>	11-DEACETOXY WORTMANNIN FUNICULOSIN SPICULISPORIC ACID
<u>Trichoderma harzianum</u>	IOSNITRINIC ACID*

* recognised as mycotoxins

APPENDIX 1

Search Strategy Used

Set	Items	Description
S1	5328	MYCOTOXIN
S2	16258	AFLATOXIN
S3	195	DIHYDROXYFLAV?
S4	736	DIACETOXYSCIRPENOL
S5	2352	OCHRATOXIN
S6	238	LUTEOSKYRIN
S7	0	EPOXY(W)TRICOTHECENE
S8	1226	STERIGMATOCYSTIN
S9	172721	TOXIN? ?
S10	2352	T(2W)2(2W)TOXIN
S11	2094	ZEARALENONE
S12	36	TRICOTHECENE
S13	394	RUBRATOXIN
S14	1506	PATULIN
S15	22846	S1 OR S2 OR S3 OR S4 OR S5
S16	173438	S6 OR S7 OR S8 OR S9 OR S10
S17	3802	S11 OR S12 OR S13 OR S14
S18	183474	S15 OR S16 OR S17
S19	1276181	ENZYME? ?
S20	261508	MANUFACTUR?
S21	2117	S19 AND S20
S22	80623	DEEP
S23	885707	CULTURE? ?
S24	2056	S22 AND S23
S25	4156	S21 OR S24
S26	77	S18 AND S25

The effect of the above strategy is that a reference is printed out if it contains one or more of the toxin keywords (S1 - S14) AND either Enzyme Manufacture OR Manufacture of Enzymes OR Manufacturing Enzymes etc., OR Deep Culture OR Deep Cultures. This gives a fairly wide coverage without overproducing results which swamp out relevant references and waste time, money and effort.

APPENDIX 2 - FILE SEARCHED

Files searched	Host	Major Journals Covered
Biotechnology	Orbit	Derwent Biotechnology Abst.
Current Awareness in Biotechnological Sciences	Orbit	Current Advances in Bio- technology Current Advances in Microbiol. Current Advances in Molecular Biol. Current Advances in Cell + Dev. Biol. Current Advances in Toxicology and many more
Biosis Previews	Dialog	Biological Abstracts
EMBASE	Dialog	Abstracts & Citations from 4000 worldwide Biomedical Journals
International Pharmaceutical Abstracts	Dialog	500 Pharmaceutical, medical + related Journals
Life Sciences Collection	Dialog	Industrial + Applied Microbio- logy, Microbiological abstracts
Chemcial Exposure	Dialog	Databank
Martindale on line	Dialog	Databank
Medline	Dialog	Index Medicus (3000 Internatio- nal Journals)
Occupational Safety & Health	Dialog	400 Journals 70,000 monographs
Chemical Regulations & Guideline system	Dialog	US Federal Databank on controlled substances
Drug information full text	Dialog	
Agrochemicals Handbook	Dialog	
CA Search	Dialog	Chemical abstracts
Merck index on line	Dialog	Merck index

Annex 7.1.4

Pages 000137-000142 have been removed in accordance with copyright laws. The removed reference is:

FAO/WHO (1990) Toxicological evaluation of certain food additives.
The 35th meeting of the Joint FAO/WHO Expert Committee on Food Additives, World Health Organization, Geneva

Annex 7.2

SAFETY EVALUATION using the PARIZA & JOHNSON DECISION TREE of Acid lactase from a genetically modified strain of *Aspergillus niger*

Introduction

The “Decision Tree for evaluation of the relative safety of food and food ingredients derived from genetically modified organisms” from the International Food Biotechnology Council (IFBC) was published in 1990¹. This publication was an extension based on an earlier publication by Pariza and Foster in 1983². More recently, an update of the 1991 IFBC Decision Tree was prepared by Pariza and Johnson and is published in the April issue of the Regulatory Toxicology and Pharmacology of 2001³.

The enzyme preparation of acid lactase from a genetically modified strain of *Aspergillus niger* TOL-54 was evaluated according the Pariza and Johnson Decision Tree. The result is described below.

Decision Tree

1. Is the production strain genetically modified?
YES
The strain *Aspergillus niger* TOL-54 is derived from host ISO-528, which is a genetically modified strain (GMO self-clone) derived from the DSM GAM lineage of *A. niger* strains.
~~If yes, go to 2. If no, go to 6.~~
2. Is the production strain modified using rDNA techniques?
YES
~~If yes, go to 3. If no, go to 5.~~
3. Issues related to the introduced DNA are addressed in 3a-3e.
- 3a. Do the expressed enzyme product(s) which are encoded by the introduced DNA have a history of safe use in food?
YES
~~If yes, go to 3c. If no, go to 3b~~
- 3b. Is the NOAEL for the test article in appropriate short-term oral studies sufficiently high to ensure safety?
NA
~~If yes, go to 3c. If no, go to 12.~~
- 3c. Is the test article free of transferable antibiotic resistance gene DNA?
YES
~~If yes, go to 3e. If no, go to 3d.~~

¹ IFBC (International Food Biotechnology Committee), Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification, Regulatory Toxicology and Pharmacology. Vol. **12**:S1-S196 (1990).

² Pariza M.W. and Foster E.M. J. Food Protection Vol. **46**. (1983), 453-468

³ Pariza M.W. and Johnson E.A. Reg. Toxicol. Pharmacol. Vol. **33** (2001) 173-186)

- 3d. Does (Do) the resistance gene(s) code for resistance to a drug substance used in treatment of disease agents in man or animals?
NA
~~If yes, go to 12. If no, go to 3e.~~
- 3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?
YES
 If yes, go to 4. ~~If no, go to 12.~~
 The final production strain is complete marker gene free and devoid of any uncharacterized heterologous DNA.
4. Is the introduced DNA randomly integrated into the chromosome?
NO
~~If yes, go to 5. If no, go to 6.~~
 The introduced DNA is targeted when integrated.
5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects, which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?
NA
 Nevertheless, the strain has been analyzed with respect to its potential to produce secondary metabolites, including mycotoxins. The strain TOL-54 showed no potential to produce secondary metabolites or mycotoxins of importance in food. This was supported by analyses on broth and concentrated UF samples (#55724).
 If yes, go to 6. ~~If no, go to 7.~~
6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?
YES
 Many strains of this safe strain lineage exist, for which safety data are available, that can be or have been tested through the P&J Decision Tree evaluation scheme.
If yes, the test article is ACCEPTED. ~~If no, go to 7.~~
7. Is the organism nonpathogenic?
NA
~~If yes, go to 8. If no, go to 12.~~
8. Is the test article free of antibiotics?
NA
~~If yes, go to 9. If no, go to 12.~~
9. Is the test article free of oral toxins known to be produced by other members of the same species?
NA
~~If yes, go to 11. If no, go to 10.~~
10. Are the amounts of such toxins in the test article below levels of concern?

NA

~~If yes, go to 11. If no, go to 12.~~

- 11 Is the NOAEL for the test article in appropriate oral studies sufficiently high to ensure safety?

NA

~~If yes, the test article is ACCEPTED. If no, go to 12.~~

12. An undesirable trait or substance may be present and the test article is not acceptable for food use. If the genetic potential for producing the undesirable trait or substance can be permanently inactivated or deleted, the test article may be passed through the decision tree again.

NA

Annex 7.2.1

WORKING GROUP ON CONSUMER ALLERGY RISK FROM ENZYME RESIDUES IN FOOD

AMFEP

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Copenhagen, August 1998

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Summary

In recent years, claims have been made by the media and some consumer organisations that enzyme residues in bread and other foods can result in allergic responses in the consumers of that food.

AMFEP established an Expert Group to evaluate whether residual enzymes in foods are an allergy risk for consumers. The Expert Group was asked to investigate existing scientific data and to report the results of the findings.

The main questions were whether enzymes in, for example, bread can sensitise a consumer of the bread, and subsequently if the presence of the enzyme residue could induce symptoms of allergy.

A further question was if a person with existing allergy to common allergens could develop allergy symptoms upon eating foods containing residual enzymes by cross reaction. This is not uncommon in the case of food allergy.

The literature survey was made to search for general food allergy, epidemiology and to find cases of food related enzyme allergy. In addition a survey of enzyme producers' files was carried out to look for adverse reactions to food enzymes.

High daily doses of industrial enzymes in are prescribed for patients with insufficient function of the pancreas. The literature on adverse events was reviewed and telephone interviews were undertaken with authorities and university hospital departments to check if experience of enzyme related gastrointestinal allergy were observed but not published.

Studies of common food allergy indicate a relatively low prevalence of about 2% of populations in Europe and the United States. There is however, a significant discrepancy between the perception of being allergic to foods (15%) and those that can be verified as food allergy (2%).

Yet, there are no firm data of the doses required to sensitise a person via the gastrointestinal tract, but the doses required to induce sensitisation seem to be very high. Indeed, patients with insufficient enzyme production of the pancreas need to take industrial enzymes in doses 100.000 - 1 million times higher than the amounts found in food.

There are no published cases of people that have been sensitised by the ingestion of food with residual enzymes, and even people who ingest high daily doses of enzymes as digestive aids are not reported to have gastrointestinal allergy to enzymes, even after many years of daily intake.

There are a few case histories of people who had reactions to papain, extracted from the papaya fruit. Papain in powder form is used as a meat tenderiser in some countries. It is unclear if the sensitisation in these cases occurred by inhalation of the powder or by ingestion of the meat with the papain.

One case history described a person who reacted with hay-fever upon eating a lactase tablet. This case was incomplete in describing the possible source of sensitisation.

There are 2 cases of people with baker's asthma and allergy to α -amylase, and wheat flour who developed symptoms after the ingestion of bread. The symptoms were somewhat more pronounced after bread prepared with α -amylase than bread without. One case with occupational allergy to α -amylase reacted upon ingestion of a very high test-dose of pure α -amylase, but not at lower doses. Four other persons with occupational α -amylase allergy did not react at any dose.

The question of cross reactions between common moulds and enzymes produced in related moulds was described in a double blind placebo controlled food challenge study of asthma patients with allergy to *Aspergillus fumigatus*. This mould is closely related to *Aspergillus oryzae* and - *niger* which are used for the production of industrial α -amylase. None of the test persons could be challenged to elicited symptoms by eating bread prepared with enzymes.

The expert group concludes that there are no scientific indications that the small amounts of enzymes in bread and other foods can sensitise or induce allergy reactions in consumers.

Employees with respiratory occupational enzyme allergy should be informed that in rare cases, symptoms may be induced by ingestion of food with residual enzymes. Enzyme residues in bread or other foods do not represent any unacceptable risk to consumers.

1.0. Introduction

Since the late 80's, and particularly since 1992 it has been repeatedly claimed that enzyme residues in foods may represent a hazard to consumers in the form of allergies, and that a certain percentage of the population are at risk of having allergic reactions to enzymes in bread and other foods.

In particular it has been claimed that consumers were at risk of developing severe allergy symptoms caused by α -amylase. The public was somewhat alarmed and there have been complaints, questions and other reactions of concern to bakers and other suppliers.

The media's interest was based on results from a study by Schata¹, published only as a 1/2-page abstract which does not allow for scientific evaluation.

However the issue was effectively raised within the public, and industry had no data with which to make a response.

Since 1992, the issue of allergy risk in consumers have emerged from time to time on television in the TV and the printed media. The general issue as it has emerged over these years is that there is a concern in the public that enzymes are unsafe, and as far as the bakers and the flour improvers are concerned, require and request data to oppose the allegations.

An additional concern is the possible cross reaction between enzymes produced by fermentation of certain moulds which may be related to common moulds. In theory, a person with a preexisting allergy to *Aspergillus sp.* might react to enzymes from e.g. *Aspergillus niger* or *A. oryzae*.

2.0 Background

2.1 General

In the public mind there is some confusion about the frequency of allergy, and in particular on food allergy. However, in the scientific community there seem to be consensus of the following:

- The frequency of common allergy (all allergies included) is 20 - 30%, in most populations around the world. The figure is increasing. Part of the increase may be due to higher awareness and improved diagnostic methods, however, a true increase cannot be ruled out.
- The frequency of occupational allergy in bakers is 8 - 27%. About 30 - 35%, of the bakers with occupational allergy to flour have an additional respiratory allergy to α -amylase and/or other baking enzymes.
- There is a reasonably good documentation of the frequency of food allergy in the general population at 1 - 2%. However, the frequency of perceived food allergy in the general population is 12 - 16%
- Food allergy does not differ from inhalation allergies with regard to the biological mechanisms taking place in the immune system. Any 'true' allergy is based on **a l l e r g y a n t i b o d i e s (I g E)**. Allergy antibodies are produced by the white blood cells called lymphocytes after the allergen has been introduced to these cells by inhalation or by ingestion. This process is called 'sensitisation'.
- Sensitisation then, is merely the event of the body recognising the foreign allergenic protein and reacting to it by producing allergy antibodies specifically recognising the particular allergen.
- Sensitisation is not a disease.
- It only becomes an allergic disease if the person develop symptoms related to exposure to the particular allergen.
- Not all sensitised people exhibit symptoms of allergy have allergy-symptoms.

2.2 Occupational respiratory allergy

allergy caused by inhalation of airborne particles of proteins, incl. Enzymes

Fungal enzymes, bacterial enzymes and extracted plant and animal enzymes are equally capable of inducing respiratory allergy - Papain and Bromelain^{2,4}, Trypsin⁵, protease's from the skin yeast *Candida albicans*⁶, from bacteria/ subtilisins^{7,8}, fungal amylases^{9,10}, bacterial amylases¹¹, fungal hemicellulases¹², lipases¹³, xylanases and cellulases^{14,15} are all examples of industrial enzymes known to induce allergic sensitisation and respiratory occupational allergy. This is a feature characterised by highly purified enzyme protein products rather than the origin or the methods of production.

They all share the structural and biological properties that may cause sensitisation when inhaled.

The classical food allergens are also capable of inducing respiratory allergy when they are brought into a dust- or aerosol form and inhaled. Soya¹⁶, eggs^{17,18}, milk¹⁹ and fish²⁰ are just examples. Soya may be one of the best described examples of epidemic inhalation allergy to an allergen also well recognised as a food allergen²¹.

3.0. Food allergy

3.1. Allergy caused by ingestion of proteins in foods

Eight percent of children under 3 years of age are allergic to food²². In, and in this age group, milk, egg, fish and soya are examples of common allergens. Many of these allergies disappear with age, but food allergy is seen also in older children and in adults. The overall frequency of verified food allergy is 1 - 2% of the population²²⁻²⁵.

Food allergy is the adverse reaction to food characterised by allergic sensitisation to food proteins and elicitation of symptoms by ingestion of the same food proteins.

Symptoms

The symptoms of food allergy are gastrointestinal with vomiting and diarrhoea, sometimes accompanied by urticaria, asthma or hay-fever. Generalised very severe reactions occur in rare cases.

Many food allergies are very mild, with symptoms of itching and burning sensation in the mouth. This is also a feature of most of the well known cross-reactions between common inhalation allergens and foods. An example can be found in patients with a birch pollen allergy who also react to e.g. fresh apples, without having a specific allergy to apples. Another well known cross reaction is that of latex and bananas. There are a number of such cross reactions between common pollen allergens and certain foods.

Types of food allergens

Examples of 'true food allergens' are proteins in milk, egg, soya, wheat, fish, nuts and, peanuts and a few more. There are others, but only about 10 food allergens account for more than 95% of severe cases. However the list of food allergens is extremely long and a large number of food allergens only give rise to allergy in sporadic cases.

The common features of food allergens are largely shared by those of respiratory allergens. However, foods are very often treated by cooking and other physico-chemical means that may destroy part of the protein structure and thereby its allergenic properties.

Properties of food allergens

The molecular weights of allergens are typically in the range of 10 -70 (90) kDa.

They have a number of 'epitopes', i.e. sequences of 8 - 16 amino acids. These are the structural 'units' which can be identified by the immune system and lead to production of specific IgE (sensitisation). In the sensitised individual the specific IgE readily recognises the epitopes on the particular protein, resulting in allergy symptoms. Some of these epitopes are described in literature²⁶⁻²⁸.

Food allergens are stable to digestion and most also to heating by cooking, and in most cases, food allergens can represent a very large proportion of the food itself. Enzymes are not well described with regard to neither their fate after ingestion nor their allergenic properties after cooking.

The TNO Institute performed a study⁵⁸ on native α -amylase from *Aspergillus oryzae* in a gastrointestinal model simulating the physiological events in the stomach.

The results indicate that about 92%, of the epitopes of the α -amylase are destroyed and about 8%, of the epitopes on the α -amylase are intact at the delivery from the stomach to the duodenum.

However, it can be expected that the proteolytic pancreatic enzymes will reduce even further, the remaining 7 – 8%, of the α -amylase during the passage through the duodenum.

Doses at which food allergy occurs

The doses and other conditions necessary to sensitise an individual are not well known. It is believed that the sensitising doses must be considerably higher than doses required for elicitation of symptoms in patients already sensitised. There are many examples of sensitised people reacting to trace amounts of allergens in the food - some of them with fatal outcomes.

It is therefore understandable that there is some focus on hidden allergens like traces of milk, nuts and peanuts in other foods.

Steinman²⁹ wrote a leading article in the August 1996 issue of J. Allergy Clin. Immunol. regarding hidden allergens in food. It is representative of the concern in the medical profession and in the public. He suggested a number of preventive measures including labelling in clear language. His article does not mention enzymes.

Food produced by GMO's

Genetically Modified Organisms (GMO's), and enzymes produced by GMO's have raised concern in general and also specifically for enzymes used in food processing.

Scientists in the fields of gene technology³⁰⁻³³ and allergy seem to agree that gene technology and the results thereof expressed in foods should not cause concern with regard to allergy risk. However, gene technology does bring about new proteins, and it is important to be aware that some of these new proteins may be allergenic.

Genetically modified proteins may, or may not share allergenic properties with traditional allergens. This would relate to the nature of the protein as it does in all other circumstances, and there are no examples of involuntary (or voluntary) changes of allergenicity of proteins in food.

A possibility may be that in the future, gene technology may be used as a tool to produce less allergenic proteins. This might be a future example of voluntary change of allergenicity.

Enzymes produced by GMO's have been on the market in some countries for many years. Enzyme producers have not experienced any difference in allergenicity of these enzymes as compared to traditional extracted or fermented enzymes. They appear to have the same sensitising potential as are capable of sensitising exposed employees at the same rate as traditional enzymes.

3.2. Epidemiology of Food Allergy

In a survey of 5000 households in the USA carried out in 1989, 1992 and again in 1993²⁵ it was found that 13.9 -16.2% of the households reported at least one member to be allergic to foods.

A study of food allergy in a random sample of 1483 adults in Holland²³ showed that 12.4% reported allergy to foods, but by controlled tests only 2.4% could be confirmed by Double Blind Placebo Controlled Food Challenge (DBPCFC).

In Spain, 3034 patients from the outpatient allergy clinics at two hospitals were tested for food allergy²⁴. The patients were tested by skin prick, RAST and open food challenge. They found 0.98% positive to one or more foods.

When looking at food additives, the same pattern emerges. In a survey of a population sample in the UK, 7% claimed to have reactions to food additives. Double blind challenge tests could verify only 0.01 - 0.23% to be true reactions to food additives³⁴.

The frequencies of confirmed food allergy in different countries in Europe and the USA are quite uniform at 1 - 2.5% of the populations.

A number of explanations to the discrepancy of perception and verified cases has been offered. There are indications that the public attribute a number of conditions to 'something in the food' and consider themselves allergic without ever having it tested.

A certain number of perceived food allergy may be induced by members of the medical profession, conducting less efficiently controlled test programs. In some cases, patients are declared food allergic solely based on skin prick tests -which may well over-diagnose food-reactions. High focus on food allergy in the media combined with personal and psychological conditions may also play a role. Actually some specialists in food allergy consider the psychological disorders the most important differential- diagnosis from food allergy.

A diagnosis must rest upon a combination of a medical history and objective tests to confirm or reject the tentative diagnosis. In the field of food-related allergies, the diagnostic test systems have been difficult to establish. However, the Double Blind Placebo Controlled Food Challenge (DBPCFC)^{35,36}, is the method of choice to confirm or reject indications of food allergy that may derive from the patient's perception and in many cases also from skin prick testing.

The experience from food allergy centres is that objective test programs to confirm or reject a suspected 'food allergy', requires skin- and blood tests and up to 6 placebo controlled challenges to be reliable.

Therefore a diagnosis of food-related allergy, based solely on medical history and a skin prick test is not good clinical practice and must be regarded un-ethical

3.3. Enzymes in food

In theory, enzyme sensitisation and allergy symptoms may be induced by direct ingestion of consumer products containing enzyme residues may occur

The tendency in recent years to focus on allergy and food allergy in particular may explain part of the marked discrepancy between the public perception of allergy to food - and the relatively few cases that can be verified in controlled clinical tests.

Papain is relatively widely used as a meat tenderiser, often supplied in a powder form to apply to the meat before cooking.

In 1983 Mansfield and co-workers³⁷ published a case story of a person who had allergy symptoms after ingestion of papain used as a meat tenderiser. - Later, in 1985 they reported a study of 475 patients³⁸ with allergy of which 5 had a positive skin prick test to Papain.

The 5 papain positive were subjected to oral challenge with papain and all had positive reactions to the challenge.

Unfortunately, the challenge was only single blinded, and there is no report of occupational exposure or the use of powdered meat tenderisers that may have caused respiratory sensitisation.

In one other case story by Binkley³⁹, described below in the section 3.6.2, it can't be totally excluded that sensitisation took place by ingestion of a food product containing relatively high amounts of industrial produced enzymes.

A recent review by Wüthrich⁴⁰ of enzymes in food concluded that orally ingested enzymes are not potent allergens and that sensitisation to ingested enzymes is rare as is also the case of reactions to bread in bakers with occupational allergy to enzymes.

The member companies of AMFEP have not registered, experienced or heard of consumers that have become sensitised to enzymes or enzyme residues in consumer products by ingestion.

It has not been possible to verify the claims in the media of such cases, and they seem as yet un-substantiated as examples of enzyme allergies in consumers. The patients presented and the symptoms and tests described are not documented, merely describing sensations and feelings, however presented as facts.

A large proportion of adverse reactions to food must be ascribed to digestive disorders such as intolerance to for example gluten and lactose, which are not allergic reactions.

3.4. The Theory of cross reactions

people sensitised with common moulds might react to enzymes produced in related moulds

The theory that people with allergy to common moulds which are related to those used for the fermentation of enzymes might react to enzyme residues in food was one of Schata's¹ claims and was given relatively high coverage in the media.

The theory could not be readily rejected as cross-reactions are relatively common in allergy. A number of food allergy reactions are merely cross reactions than caused by primary sensitisation.

The most commonly used moulds for fermenting enzymes are *Aspergillus oryzae* or *A. niger*.

According to the theory, people with allergy to *Aspergillus*-moulds would be a high risk population. *Aspergillus* allergy occurs in less than 0.5%, of the population.

A study by Cullinan⁴¹ was conducted with the objective of testing if patients with a well-documented allergy to the widely distributed common mould *Aspergillus fumigatus* reacted upon the ingestion of bread prepared with enzymes of *Aspergillus* origin. The study was a double blind placebo controlled food challenge study on 17 *Aspergillus* allergic people.

The 17 test persons all had allergy antibodies to *Aspergillus fumigatus*, but in addition, 6 also reacted at the skin prick test to the enzymes produced in *A. oryzae* or *A. niger*.

Each patient was challenged with bread baked with the 2 enzymes in standard doses and with placebo bread baked without enzymes. Allergy symptoms and a number of general physiological parameters were monitored before, during and for 24 hours after the challenge.

No allergic reactions were seen upon ingestion of enzyme containing bread as compared to placebo bread.

This study clearly demonstrates that patients who must be considered at the highest risk for cross reactions to baking enzymes do not react with clinical symptoms when they eat enzyme containing bread containing enzymes.

It is a general experience that once a person is sensitised, even very small amounts of the allergen can elicit allergy symptoms.

In the case of baking enzymes it seems well documented that even patients with severe asthma caused by *Aspergillus fumigatus* did not react to the baking enzymes produced in *A. oryzae* and *A. niger*.

3.5. Food related reactions in occupationally sensitised people

The situation of possible reactions to enzymes in bread in patients with occupational allergy to enzymes

There are a few papers describing cases of allergy symptoms elicited by the ingestion of enzymes in people who have occupational allergy to enzymes:

Kanny & Moneret-Vautrin,⁴² and Baur & Czuppon⁴³ each describes one patient who since late childhood, has had asthma and occupational asthma with allergy to flour and enzymes for several years. Both patients were tested for elicitation of symptoms by ingestion of bread baked with and without enzymes. Kanny & Moneret-Vautrin's patient was tested in a blinded design, Baur's patient in an open, non-controlled programme. In both cases the result was elicitation of respiratory symptoms after challenge with bread baked with enzymes. Baur's patient also had a slight reaction to bread without enzymes, however not as pronounced as the reaction after the enzyme containing bread.

Losada et al⁴⁴ investigated occupational allergy to α -amylase in a pharmaceutical plant and found a number of employees sensitised to α -amylase. None reported reactions related to ingestion of bread. Five patients, all positive to α -amylase were given oral doses of native α -amylase in doses up to 10 mg.

At this dosage, one of the 5 test persons reacted with respiratory- and generalised allergy symptoms. Four did not react.

Baur et al⁴⁵ described the possible background for consumer sensitisation to α -amylases in bread. 138 subjects, of which 98 were allergic, and 11 bakers with occupational allergy were tested. The bakers reacted to α -amylase as may be expected. None of the atopics and none of the control persons reacted to skin prick test with α -amylase. Two atopics had weak RAST to native α -amylase and one reacted also to heated α -amylase. Reactions to other related compounds, for example *Aspergillus* was not tested.

Tarlo and co-workers⁴⁶ reported results of testing for papain allergy in 330 allergy patients. - Seven had positive RAST and Skin prick test but none of them had any gastrointestinal or other allergic symptoms to papain.

The elicitation of gastrointestinal symptoms upon respiratory sensitisation is also reported for flours. One example is reported by Vidal et al⁴⁷ and describes a man with occupational asthma after exposure to flours and other grain dusts. He was sensitised to barley, and experienced gastrointestinal reaction upon ingestion of foods and beverages made from barley.

Enzyme producers and other companies handling concentrated enzymes do see cases of employees being sensitised to baking enzymes. These would be the people at the highest risk of reacting to enzyme residues in bread.

However, none of the members of AMFEP had any reports of sensitised employees who had experienced allergy symptoms in connection to ingestion of bread, and there are no reports of α -amylase sensitised employees avoiding bread.

Cases of people with occupational allergy to flours and food-related reactions to ingestion of flours/bread do occur. One case report describes a person with asthma to barley dust and also with reaction to beverages and foods produced from barley.

The conclusion from these reports of people with pre-existing occup. allergy to α -amylase is:

- Allergic reactions after ingestion of enzyme containing foods are described in 3 individuals.
- The 3 cases are people with definite occupational respiratory allergy to flour and an additional sensitisation to α -amylase. It means they are most probably sensitised by inhalation of flour dust and enzyme dust and not by eating bread or other foods with enzyme residues in it.

3.6 The consumption of enzymes for medical purposes and as digestive aids:

Many people around the world eat enzymes for medical purposes or for convenience as digestive aids.

In many countries enzymes are used routinely as digestive aids by healthy people. The number of people in the world, frequently eating enzyme preparations must be counted in millions.

A number of diseases require the daily addition of enzyme preparation to the food to compensate the patient's insufficient production of digestive enzymes.

3.6.1. Medical uses:

Medical use of enzyme preparations are subject to clinical trials, the results of which are normally reported to the health authorities, and such adverse effects are described in the pharmacopoeia/registry of drugs.

Patients with chronic pancreatitis suffer from insufficient production of digestive enzymes from the pancreas. They are dependent on daily intake of enzymes, some of these produced from *Aspergillus* and other moulds, some extracted from animal glands. The doses of these enzymes are in the order of gram's a day. - we have not been able to identify published documentation of allergy to enzymes in these patients, and the drug registry's does not even mention allergy as an adverse effect.

Proteolytic enzymes and mixtures of different enzymes are commonly used for treatment of a number of physical lesions and also for a number of more special conditions⁴⁸⁻⁵⁰.

The enzymes are administered in the form of tablets with mixtures of enzymes and in doses of 6 to 600 mg per day, in some cases several times more.

We have not been able to find any evidence of sensitisation or allergy symptoms caused by the ingestion of enzymes from these enzyme preparations. One example is the use of enzymes given as tablets for the treatment of non-articular rheumatism. Uffelmann⁵¹ describes a double blind study of 424 patients, of which 211 received enzyme treatment. The daily doses of the mixed enzyme preparations was 240 mg Lipase, 240 mg Amylase, 1,44 g Papain, 1,08 g Bromelain and 2.4 g Pancreatin,. This dosage was given for 8 weeks and no serious adverse effects and no allergy reactions were reported.

Patients with Cystic Fibrosis suffer a hereditary disease characterised by severe lung symptoms and insufficient production of digestive pancreatic enzymes. They too are dependent of daily intake of grain-doses of enzymes. - There are a few reports of parents

and hospital staff who have become sensitised by inhalation of dust from these enzyme preparations⁵²⁻⁵⁴. This of course might also happen to the Cystic fibrosis patients when they handle the enzyme preparations themselves. However no cases of enzyme allergy in Cystic Fibrosis patients have been described, but there are reports of allergy to common food allergens⁵⁵.

An informal telephone survey on unpublished cases of enzyme allergy to European Cystic fibrosis Centres, resulted in only one possible case. The patient was a boy who reacted with vomiting after administration of the enzyme preparation containing amylase, protease and lipase. - The enzyme treatment had been stopped because of suspected allergy to the enzymes. However, testing for specific allergy antibodies by Maxisorp RAST⁵⁶ did not confirm sensitisation to any of the enzymes. Challenge tests have not been performed⁵⁷.

3.6.2. Digestive aids one possible case of allergy to digestive aid enzymes

In some cultures the use of digestive enzymes after large meals is very common. Enzymes for this purpose are 'over the counter' (OTC) drugs. We have found no studies of possible allergy to enzymes in these populations. That may be irrelevant if no-one ever thought of the possibility that enzymes might be the cause of allergic symptoms had not been considered. - However, with millions of people using enzymes frequently, some cases of adverse effects in the form of allergic symptoms would be expected to emerge and be described in the literature. In most patients with allergic reactions, symptoms would appear immediately or very shortly after the intake.

Binkley³⁹, described a case of allergic reaction to ingested lactase. This patient had a respiratory allergy with positive skin prick test reaction to *Aspergillus sp.*

He had had two incidents with allergic reactions in the form of swelling and burning of Lactaid tablets. The lactase was produced from fermentation of *Aspergillus oryzae*. Skin prick test with extracts of Lactase tablets gave a very strong positive reaction. He had not taken Lactaid tablets previous to the first experience of symptoms, but he had taken milk products containing lactase from *Saccharomyces fragilis* and from *Kluyveromyces lactis*. Although highly unlikely, it may be speculated if these may cross react with Lactaid. In this case it seems unlikely that sensitisation was caused by the Lactaid tablets as the symptoms appeared the first time he ever took Lactaid. It could be a 'cross reaction' based on sensitisation to yeast-produced lactase and symptoms elicited by the ingestion of Lactaid. Another possibility may be a cross reaction from his pre-existing *Aspergillus sp.* allergy.

This case may be regarded a possible but not verified case of oral sensitisation to enzymes in food.

A few other consumers have claimed allergy to these OTC drugs but thorough testing has not verified allergy to enzymes in any of these cases.

With the background of the very high awareness of food related allergy in the populations, the widespread use of digestive aid and medical uses of enzymes should have attracted interest if allergy to ingested enzymes were of importance. However, up to now, only the single case mentioned above have been described.

To evaluate the risk of sensitisation from ingestion of enzymes and eventually experience of symptoms, we are aware of only the one case that may have become sensitised by ingestion.

This has to be related to the total number of people world-wide who ingest enzymes for short periods of time as part of a medical treatment, and to those who are dependent of daily intake of high amounts of digestive enzymes.

4.0. Conclusion

The working group has studied the available literature on these subjects and came to the conclusion that from a scientific point of view there is no indication that enzyme residues in bread or in other foods may represent an unacceptable risk for consumers.

Lack of scientific data is not evidence of lack of risk, and the working group realises that evidence of 'no risk' is extremely difficult or impossible to generate.

The group wish to stress that a 'zero-risk' can never be proved by science, and it must be anticipated that even an extremely low risk (e.g. 1 in 50 or 100 millions) of verified allergy to enzymes in food may well be perceived as a significant and unacceptable risk by the public in which more than 10% believe they are allergic to food.

Scientific data are of high value as the credible background for promotion to the public, to trade organisations and individual customers and for an ongoing dialogue with opinion leaders and consumer organisations.

It is the opinion of the group that many cases of perceived allergy to enzymes may be attributed to insufficient diagnostic procedures employed by members of the medical profession.

A minimum requirement for establishing a diagnosis of food related enzyme allergy should be a well conducted DBPCFC.

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Annex 7.2.1.A



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POSITION PAPER

ETA Position On Food Allergen Labeling of Microbially Derived Enzymes Under FALCPA as it Applies to Fermentation Media Raw Materials

It is the position of the Enzyme Technical Association (ETA) that microbially derived enzymes do not fall within the scope of the Food Allergy Labeling and Consumer Protection Act (FALCPA) and that labeling for food allergens is not triggered by the use of a microbially derived enzyme preparation. There may be other reasons why a manufacturer labels a food product with regard to allergen content, but the use of a microbially derived enzyme preparation is not a reason for such labeling.

Enzymes are not one of the eight major allergenic foods, often referred to as the big 8, so they do not fit within the first requirement of FALCPA. In addition, microbial enzymes are not byproducts of nor are they derived from the major food allergens. Although enzymes are not major food allergens,¹ many enzymes are produced with microorganisms and the nutrient media used to feed these microorganisms may contain protein from one or more of the major food allergens. The enzymes are not derived from raw materials containing major food allergens, but rather are obtained from the microorganisms which are used to produce the enzyme proteins. In other words, enzymes obtained from fermentation are directly derived from microorganisms fed on media that may include protein obtained from one or more of the major food allergens. Proteins and other nitrogenous material are consumed by the microorganisms for cell growth, cell maintenance, and production of enzyme protein. It is the intent of the enzyme manufacturer to supply enzymes; therefore it is critical that the ratio of nutrient to enzyme yield is carefully controlled. It is also the intent of the manufacturer that these raw materials are added to the fermentation as food to be consumed by the microorganism and are not added as formulation ingredients.

In arriving at its position ETA also considered that:

- The regulatory agencies in the EU and Japan have determined that enzyme preparations are not required to have allergen labeling for the raw materials used in the fermentation process. Indeed, the European Commission's Health & Consumer Protection Directorate General has clearly stated that enzymes

¹ To the extent the enzyme producer uses an allergenic material, such as wheat flour diluent in the final product formulation, labeling may be required.

are outside the scope of the Directive 2003/89/EC which amended the EU Food Labelling Regulations.

- Enzyme broths are normally processed to separate biomass and fermentation materials from the enzyme, to concentrate the enzymatic activity, and formulated to achieve a uniform and stable enzyme product.
- The unique role of enzymes in food processing is as a catalyst. Due to the specific nature of enzymes, only small amounts are required to make desired modifications to the property of a food.
- Many enzymes do not become a component of the food ingredient or final food. Some enzymes are used in an immobilized form or are denatured during processing. Further, processing of the food ingredient after the enzyme catalyst has performed the expected function often reduces or eliminates the enzyme from the product.
- ETA has made an extensive review of the published scientific literature and has found no reports that even suggest there has been an allergenic reaction to a component of the fermentation media which was used to feed the microorganism that produced the enzyme.

The above position paper and accompanying report were provided to FDA on September 12, 2005 and to date ETA has received no comment.

Annex 7.2.1.B

EXPERT OPINION STATEMENT
FOOD ALLERGY RESEARCH & RESOURCE PROGRAM
UNIVERSITY OF NEBRASKA

**Testing of Microbially Derived Enzymes for Potential Allergens from
Fermentation Media Raw Materials**

August 13, 2013

Prepared by: Steve L. Taylor, Ph.D., Co-Director
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with assistance from Enzyme Technical Association

Microbially derived enzymes are used by food processors as additives and processing aids in a wide variety of foods. Enzymes obtained from microbial fermentation are directly derived from microorganisms fed on sterilized media¹ that may include protein sources obtained from one or more of the recognized commonly allergenic foods (e.g., milk, soybean) or from a cereal source of gluten (e.g., wheat, barley). This paper addresses the relevance of testing microbial enzymes for allergenic material from the fermentation growth media.²

It has been the long-standing position of the Food Allergy Research & Resource Program (FARRP) at the University of Nebraska that testing of the products of fermentation (with limited exceptions), including microbially derived enzymes is unreliable using enzyme-linked immunosorbent assays (ELISAs).

While various fermentation media may contain one or more of the major food allergens, the biochemical reactions that occur during fermentation result in the breakdown of the fermentation media proteins. The extent of proteolysis is dependent upon the fermentation culture and the resultant enzyme (e.g., some enzymes are proteases). As proteins are digested, the resulting amino acids, along with other

¹ Aunstrup, K., O. Andresen, E.A. Falch, and T.K. Nielsen (1979) *Microbial Technology*. (Perlman and Peppler, eds.) Academic Press, pp. 281-309.

² For this paper, FARRP's analysis is limited to microbially derived enzymes that are intended for additive and processing aid applications in food.

nitrogenous material, are consumed by the microorganisms for cell growth, cell maintenance, and production of enzyme protein.

Upon completion of fermentation, remaining fermentation media that are not consumed by the microorganism are typically separated and/or purified from the enzyme in the recovery process. Enzymes are recovered from the fermentation broth by standard chemical engineering operations, such as filtration and centrifugation, broadly used in enzyme production.^{3,4} (See Appendices for further information.) The recovery steps result in separation of microbial biomass and other fermentation solids from the enzyme, concentration of the enzyme, and removal of impurities prior to final formulation with food-grade ingredients.

Any potential residual fragments from the food allergen would be difficult to measure as there is no reliable assay. Commercial ELISAs are able to detect only intact proteins in most cases. Any peptides, even larger ones, would not likely be detected, although this possibility has not been well investigated. Results would typically be reported as below the limit of quantitation for the enzyme preparation. Further, if any residual but undetected fragments of the food allergen remain, the relevance of any such residual material to food allergenicity is unproven. Accordingly, testing of fermented product does not result in reliable or useful data.

In addition, due to the specific catalytic nature of enzymes, only very small amounts of enzymes are generally required and used by food processors to make the desired modifications to the property of a food, and therefore any *de minimis* amount of fermentation media protein that may survive the fermentation process will not pose a significant public health risk to the consumer.⁵

FARRP also notes that regulatory agencies in the European Union and Japan do not require allergen labeling of enzyme preparations for the raw materials used in the fermentation process.

³ Atkinson, B. and F. Mavituna (1991) *Biochemical Engineering and Biotechnology Handbook*. (Atkinson, B. and Mavituna, F., eds.) Stockton Press, Hampshire, pp. 1146-1158.

⁴ Kroschwitz, J.I. (1994) *Enzyme Applications in Encyclopedia of Chemical Technology*. 4th edition, Volume 9. (Kroschwitz, J.I., ed.), pp. 567-620.

⁵ To the extent the enzyme producer uses an allergen as diluent to formulate the final product, labeling for such allergen is appropriate and required under Food Allergen Labeling and Consumer Protection Act.

SUBMISSION END